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THE WEAK A ANTIGEN IN GROUPS $_3$A AND AB BLOODS*

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The most significant characteristics of the weak A agglutinogen and the variants of the A agglutinogen are the weak or negative reactions with potent anti-A serum,[7] and the stronger reactions with Group O serum [anti-A and B].[4] Dahr[3] suggests that the A agglutinin in Group O serum is qualitatively different from the A agglutinin in Group B serum. The inclusion of Group O serum, absorbed for anti-B, with the anti-A and anti-B sera in the cell typing procedure would prevent weak type A blood from being reported as Group O, and weak Group AB, as Group B, in most instances. Grave transfusion accidents can occur when such incompatible blood is given to recipients.[8] The occasional practice of giving Group O Rh negative blood in an emergency without a proper cross match can have serious consequences.[6] The donor who is classified as Group B because his weak A antigen is overlooked could cause a transfusion reaction when the A agglutinin in the serum of the Group B recipient reacts with the donor's weak A antigen. Van Loghem[11] calls these "weak" antigens "strong" antigens, because they react strongly in absorption, inhibition and elution tests. Perhaps these properties, if carried over in vivo, make these antigens strong there also. It is rather interesting that in all the recorded cases of mistyping of AB blood, the recorded cell Group was B, never A.[5**†]

Inability to demonstrate the weak A agglutinogen is also a hazard in typing the blood of newborns. Race and Sanger[10] state that the antigens in newborns are weaker than the antigens in adults. This would almost certainly result in a weak A antigen or A variant being overlooked in newborns. This possibility is further increased in Group AB newborns according to Wiener[12] because in AB blood the B antigen suppresses the A antigen. In addition, in infants there are no specific agglutinins and the cell typing cannot be checked by the serum typing.[9]

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** While a serum or reverse typing might help in most of these AB bloods with weak A antigen, it would be confusing in others, where the anti A_1 present agglutinated the Group A test cells which must be A_1.

† In our cases, the cell typings read as B and the serum typings AB. This discrepancy made us suspect that something was wrong that needed further checking.

Attempts to demonstrate the presence of the weak A agglutinogen by other sensitive technics such as the Coombs and the enzyme methods have failed. In most cases where anti-A serum obtained from rabbits immunized with Group A cells was used, the results have not been satisfactory.[13]

These attempts to demonstrate the weak A agglutinogen by some sound method of cell typing suggested the possibility of using pooled Group O [anti-A and B] serum, which had been absorbed with Group B cells or substance. A few cases are mentioned in the literature where this was attempted. Cahan[1] absorbed type O serum with B cells in his efforts to agglutinate A_x cells. Junqueira[8] used the saliva of a B secretor to absorb Group O serum. This latter method is not practical, since once the absorption is accomplished the saliva cannot be removed and remains as a permanent diluent and contaminant in the absorbed serum.

The method recommended here makes use of Group B cells. They are easily obtained; they can be washed clean in two or three washings with normal saline; and they can be removed completely from the Group O serum after absorption. The anti-B is absorbed here to prevent its masking the reaction with the weak A antigen in Group AB blood.

I. Technic for Absorbing Group O Serum with Group B Cells.

Since Group O serum is so easily available to most blood banks, it is not necessary to absorb large quantities of it at a time, unless a supply of immune Group O serum with a high anti-A titer is available.[2]

A. To 10 cc of pooled Group O serum in a small flask add an equal volume of washed, packed Group B adult human red blood cells.

B. Place in the refrigerator at 4° C. for two hours, shake occasionally, though gently, to bring more free cells in contact with the available anti-B.

C. Transfer the contents of the flask to two test tubes and centrifuge for five minutes at high speed.

D. Transfer the supernatant serum to another tube. Discard the cells.

E. To test for absorption of the anti-B, place one drop of the serum in an 8.5 x 75 mm tube, and add to it one drop of a 5% suspension of B cells in normal saline. Centrifuge for one minute. If negative, let stand in the refrigerator for an hour. Centrifuge again. If negative confirm with the Coombs test. If all tests are negative, the serum can be considered absorbed.

F. If still positive with B cells by any of these tests, a further small absorption with half the quantity of B cells used in the first absorption will be enough to complete the process.

G. The completely absorbed Group O serum is tested with 5% saline suspensions of A_1 and A_2 cells. It should be strongly positive with both.

H. The serum can be stored in the deep freeze in small aliquots for future use.

II. Technic of the Test for Weak A Antigen in AB Blood.*

A. A 5% washed suspension of the cells to be tested is prepared.

* Note: Absorption of anti-A was not done here because the phenomenon of the "weak" A antigen was treated here as a cross matching problem rather than a research project.

B. Ten 8.5 x 75 mm tubes are numbered from 1-10, and the following protocol is drawn up at the same time.

C. Of the ten tubes, tubes 1-4 are the test, tubes 5-10 the controls.

D. Test of the unknown cells:

1. 1 drop anti-B serum plus one drop test cells.
2. 1 drop anti-A serum plus one drop test cells.
3. 1 drop Group O (anti-A, B) serum plus one drop test cells.
4. 1 drop anti-A [absorbed Group O] plus one drop test cells.

Controls:

5. 1 drop absorbed Group O plus one drop A cells.
6. 1 drop absorbed Group O plus one drop B cells.
7. 1 drop absorbed Group O plus one drop O cells.
8. 1 drop anti-A serum plus one drop A cells.
9. 1 drop anti-B serum plus one drop B cells.
10. 1 drop Group O (anti-A, B) serum plus one drop O cells.

E. Centrifuge for one minute and read. If doubtful incubate for 10 to 20 minutes in the refrigerator at 4° C. and read after centrifuging for one minute.

F. The results are strong and unequivocal.

III. Results with several weak Group AB bloods

TABLE 1

	Blood Tubes	1	2	3	4	5	6	7	8	9	10
1.	Mrs. J.	++	—	++	++	++	—	—	++	++	—
2.	Mrs. L.	++	±	++	+	++	—	—	++	++	—
3.	Mr. S.	++	—	++	+	++	—	—	++	++	—
4.	Baby F. (Cord)	++	±	++	++	++	—	—	++	++	—
5.	Baby Y. (Cord)	++	—	++	+	++	—	—	++	++	—

+ is positive; — is negative; ++ is strongly positive.

IV. Conclusion

Group O serum absorbed with B cells gave positive to strongly positive reactions with Group AB bloods containing weak A agglutinogen. These bloods had given weakly positive [doubtful] or negative results with a potent anti-A serum. This technic provides a reliable method for solving the problem of the weak A antigen.

V. Summary

A method is described for the preparation of an absorbed Group O [anti-A] serum to demonstrate the weak A agglutinogen which may escape detection by potent anti-A serum.

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ENZYME CHEMISTRY AND FUNCTIONS*

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LIFE. Such a tiny word, only four letters but what a vast meaning as stated by Webster, "The quality or character distinguishing an animal or a plant from inorganic or from dead organic bodies, which is especially manifested by metabolism, growth, reproduction, and internal powers of adaptation to environment."[6] But, you are asking, where does enzyme chemistry enter the picture of life? In a sense, enzymes are the very center of life, they initiate and organize the complex chemical changes in cells which we shall term metabolism. Metabolism is divided into two classes: (1) Anabolism, those chemical reactions which build new tissue, replace old tissue, and prepare substances used as reserve food supplies or as catalysts; (2) Catabolism, reactions that result in the destruction of tissues or in the production of energy by the oxidation of foods.

Enzymes are organic catalysts produced by living cells which hasten the rate at which a given reaction will come to equilibrium. Catalysts are substances which alter the velocity of chemical reactions. An ideal catalyst, while participating in the reaction, is neither destroyed nor permanently altered. Actually, catalysts are gradually consumed by secondary reactions and seldom do they catalyze more than 200,000 times their own weight of reacting material. For this reason, they must be replaced or regenerated eventually. No energy is derived from the catalyst, so it does not alter the equilibrium point of a reversible reaction. Catalysts form intermediate, highly reactive compounds; and, when present in colloidal form, they initiate adsorption reactions. An enzyme is, in a sense, a "living" molecule; conditions or substances that destroy life, such as heat, corrosive reagents, acids, bases, poisons, and antibiotics, also destroy or inhibit enzymes.

An enzyme differs from an ordinary laboratory catalyst in that the enzyme acts specifically on a certain substance which is termed its substrate. Enzymes are divided into two biological classes: extracellular enzymes, secreted by cells; and endocellular or intracellular enzymes which normally are retained within the cells.

All enzymes that have been isolated from tissues and studied have been proved to be proteins. Enzymes are present in the digestive juices of the stomach and intestine, they are also found in the blood stream. Many enzymes are found to be inactive unless united with a heat-stable substance, called a coenzyme, which is a non-protein organic molecule. There are present in the body activators and inhibitors of enzymes. An activator is an inorganic ion which helps to speed up the reaction of an enzyme. The inhibitors of enzymes are substances which precipitate proteins from solution and may even stop all enzyme action. An example of inhibitors are the salts of (HCN) they are deadly poisons to the body because they inhibit many enzymes. This leads to death because without the action of the enzymes chemical reactions necessary for life cannot proceed.

* Read by title, 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959.

Enzymes are classed according to the types of reactions which they catalyze. They are divided into two groups: The hydrolytic enzymes, which catalyze hydrolytic reactions; and the oxidizing-reducing enzymes, which catalyze the oxidation or reduction of their substrates. All the digestive reactions of the body involve hydrolysis. The food combines with water to yield amino acids. The hydrolytic enzymes then recombine the products of digestion to build up fats, carbohydrates and complex proteins which make up the structure of living tissue.

Enzymes are greatly affected by temperature and pH. Enzymes are heat-liable proteins which undergo chemical denaturation and oxidation at higher temperatures. The optimum temperature of an enzyme is the point at which increased reactivity is balanced by increased destruction of the enzyme. Enzymes are most effective at temperatures between 38° and 45° C.

Enzymes are amphoteric colloids, and, therefore, are subject and sensitive to pH changes. The optimum pH is the pH at which an enzyme is most effective.

The function of enzymes, as stated earlier, is that they initiate and partly control almost every conceivable physiological process. Enzymes together with activators, inhibitors, coenzymes and proenzymes, which are substances that are not enzymes when first made in the body cells, but which become enzymes when they come in contact with some other substance—a kinase, control all the functions of the body. Chart 1 contains a list of a few enzymes and the organs where they are found.

CHART 1

Enzyme	Location
Ptyalin & Maltase	Saliva
Pepsin & Rennin	Gastric juice
Amylase & Trypsin & Lipase	Pancreatic juice
Glutamic Oxaloacetic Transaminase	Heart muscle
Cholinesterase	Liver
Phosphohexase	Liver
Acid phosphatase	Prostate gland
Lactic Acid Dehydrogenase	Cardiac muscle

Since enzymes play such an important role in our life, it is very important that we can perform accurate analyses or enzyme determinations. Since the demonstration of enzymic action of yeast by Harden & Young in 1905, the usage of enzyme studies in the conception and treatment of disease has accelerated tremendously.[1] Under controlled conditions the rate of an enzyme reaction is proportional to the enzyme concentration. So in the determination of blood enzymes, the basic principle is that the enzyme is allowed to decompose a substance which is known as a substrate. After a known time, the amount of decomposition is measured. The more the reaction of decomposition, the greater the concentration of enzyme. Enzyme concentrations are determined and expressed in relation to arbitrary standards of activity, using the time required to produce a definite degree of conversion of an appropriate substrate or the quantity of the preparation necessary to produce

such conversion in a specified time. The enzyme determinations that we, as medical technologists, perform today are concerned with the diagnosis, prognosis, and the observation of the effectiveness of the various forms of therapy. At this time I shall give a brief review of the more frequent enzyme determinations, together with their respective normals, functions, optimum pH and the conditions where they are found to be abnormal whether increased or decreased.

Amylase—is involved in the digestion of starch. The optimum pH of amylase in the blood is 7.0. One mg. % of reducing sugar equals 1 unit of amylase. The normal range is 80-150 Somogyi units. Amylase when found in the urine is referred to as Diastase. The normal range is up to 300 units for urine. Amylase is found increased in acute pancreatitis, carcinoma of head of the pancreas, duodenal ulcer, mumps, hyperthyroidism. It is decreased in cirrhosis of the liver, carcinoma of liver, acute alcoholism and hepatitis.

Lipase—is present in the pancreatic secretion and its function is to produce a hydrolytic action upon ingested fats. The degree of lipase activity is indicated by the amount of fatty acid liberated from a substrate of olive oil emulsion at a pH of 7.0. It is reported in terms of cc. of 0.05 N NaOH necessary to titrate the acid liberated by 1 cc. of serum. Normal range—0.0-0.3 units (Cherry & Crandall). Elevated lipase values may be found in acute pancreatitis, intestinal obstruction, and carcinoma of the pancrease.

Trypsin—is the principle proteolytic enzyme of pancreatic juice and is an integral part of the metabolism of protein. Very little trypsin passes into the blood system and therefore the assays are performed on duodenal juice.[1] Normal range—7.5-15 gm. of Nitrogen per 100 cc. of duodenal contents. Optimum pH of 8.0. Trypsin levels are decreased in fibrocystic disease of the pancreas.

Pepsin—completes the hydrolysis of proteins to amino acids in the duodenum. It is the principal enzyme of the gastric juice and its main determination is to see whether achlorhydria or achlyia is present. Optimum pH of 2.0. Pepsin is usually absent in pernicious anemia, severe secondary anemias, or following attack of gastroenteritis in children.

Lactic Acid Dehydrogenase—is determined by measuring the conversion of pyruvic acid to lactic acid. The normal range is 165-313 units/ml. A unit of activity is defined as the increments of 0.001 optical density fall per minute per milliliter. The infant level is about twice the adult level. Normal cerebrospinal fluid is up to 40 units/ml. Lactic acid dehydrogenase is increased in myocardial infarction and certain neoplastic processes, and also in acute hepatitis. The optimum pH is 8.0.

Cholinesterase—serves to catalyze the hydrolysis of acetylcholine the neuro-hormone of the nervous system. Normal range—0.4-0.88; optimum pH of 8.4. Reduced levels are found in liver disease.

Alkaline Phosphatase—Normal range—1.5-4.0 units adults, 5.0-12.0 units (Bodansky) children. The Bodansky unit of phosphatase activity is defined as that amount of activity which will liberate 1 mg. of inorganic phosphorus per 100 cc. of serum from a sodium glycerophosphate substrate solution (pH 8.6) during the first hour of incubation at 37° C. Increased values are found in rickets, hyperparathyroidism, carcinoma

of the bone, Paget's disease, multiple myeloma; decreased values are found in scurvy, chronic nephritis, and celiac disease.

Acid Phosphatase—Normal range 0.0-1.1 units (Bodansky). Optimum pH 5.0-6.0. Increased levels are noted in carcinoma of the prostate with metastases and extensive local extension.

Transaminase—is found in elevated amounts in the serum in association with several disease states, notably, myocardial and liver damage. Transamination is of critical importance because it is a step in the synthesis of many amino acids from keto acids, which are acids in which the amino function has been replaced by the keto function. Since tissue proteins are constantly being built up and broken down, one major step in protein metabolism involves amino acid synthesis, a step in which the enzyme transaminase plays its vital role. The normal range is 10-40 units.

Enzymes are not only important to the physician for the role they play in diagnosis and prognosis of disease processes, but because by using combinations of enzymes speeds the healing of infected wounds by removing exudate and necrotic debris. The enzymes which have been successfully employed by many investigators are trypsin, a proteolytic substance, and streptokinase-streptodornase, a combination of enzyme activator and enzyme produced by certain strains of streptococci.

Enzyme chemistry is a new field which is opening wider and wider every day for the medical technologist. An enzyme may be compared to a puppeteer and all the organs of the body as puppets; without the enzymes pulling the strings—life could not exist.

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RECENT OBSERVATIONS IN TUBERCULOSIS BACTERIOLOGY*

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Antituberculous chemotherapy has presented many problems in the tuberculosis bacteriology laboratory. Tubercle bacilli which have become drug resistant develop fastidious requirements for growth.[14] There is difficulty in determining the morphology of these variants. Many specimens contain drug resistant bacteria which contaminate our media. New methods must be devised to destroy these bacteria without inhibiting the growth of the tubercle bacillus.

Cultures of smear positive specimens have failed to grow.[5] New methods and thorough observations must be made.

Newly reported cases of tuberculosis have not fallen at the same rate as the dramatic fall in mortality. Greater care must be taken in culturing specimens from which very few colonies will grow.[5]

The role of the atypical acid-fast bacilli must be evaluated so that the disease causing organisms can be differentiated from saprophytes and those which do not cause disease.[3, 11]

Our laboratory has tried to find an answer to these problems. Perhaps the following remarks will benefit others who work in diagnostic laboratories.

Growth Requirements

Isoniazid-resistant strains[9] are usually deficient in their ability to metabolize certain elements in synthetic media. Substances must be added which can be used by tubercle bacilli with altered cytochrome enzyme systems.[10] Excellent results are obtained when 10% CO_2 is added.[14]

The neutralized sediment of 959 specimens of sputum or gastric washings digested with 3% NaOH was seeded in equal volumes on 4 slants of Lowenstein-Jensen medium in tubes loosely closed with plastic screw caps. The tubes were incubated at 37 degrees C. for 12 weeks and were inspected weekly for growth of tubercle bacilli. Two tubes were incubated in air and two were incubated in a sealed cabinet to which was added 10% CO_2 by volume at atmospheric pressure.

Growth of acid-fast bacilli was obtained from 233 specimens. Atypical acid-fast bacilli were found in 39; they grew rapidly and equally well in both 10% CO_2 and aerobic incubators.

Tubercle bacilli were recovered from 194 specimens. Of these, 116 were susceptible to isoniazid (0.1 mcg./ml.). Twenty per cent of the cultures grew in CO_2 and not in air. Some grew more rapidly in CO_2 than in air. A significantly greater number of colonies were found under CO_2 with 34 additional cultures; 20 cultures showed only tiny colonies in air but large colonies in CO_2.

There were 78 isoniazid-resistant ($>$ 5mcg./ml.) strains of tubercle bacilli. Ten of these strains grew only in the presence of added CO_2; eight of the ten lacked detectable catalase activity.[16] These resistant bacilli often showed their earliest growth in CO_2, particularly those

*A.S.M.T. 3rd Award, 1959. Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959.

strains with no catalase. Moreover, 17 additional strains of resistant tubercle bacilli showed significantly more colonies under CO_2 and 12 strains had strikingly larger colonies. In no case did aerobic conditions favor the growth of isoniazid-resistant tubercle bacilli.

Many isoniazid-resistant tubercle bacilli do not develop the characteristic "rough" morphology when grown without CO_2. Instead, the colonies are smooth like those of atypical acid-fast bacilli. A rapid aid toward identification may be to perform the catalase test.[8] Negative to 3 plus catalase activity indicates that the organism is probably Mycobacterium tuberculosis. In contrast, saprophyte radish which can have "rough buff" colonies similar to tubercle bacilli has strong catalase activity (5 plus).

Peizer's agar media with beef extract proves to be a good second media because growth occurs early. Five out of 15 lung tissue cultures grew on Peizer's media but failed to grow on Lowenstein-Jensen media.

The rate of contamination was high when sputums were inoculated on Peizer's media. Colony morphology often had to be checked by subculturing to an inspissated egg media.

Contaminants

During a 12 month period 8500 sputum, gastric and urine specimens were cultured with 3% NaOH digestion and inoculated on 2-4 tubes of Lowenstein-Jensen media. All tubes were contaminated in approximately 5% (443). Of the 5%, the contaminated cultures consisted of:

Gram variable spore forming rods	32%
Paracolon bacillus	15%
Pseudomonas	13%
Escherichia coli	11%
Proteus	6%
Hemolytic staphylococcus aureus, coagulase positive	5%
Aerobacter	4%
Fungi	3%
Alkaligenes	2%
Other bacteria	9%
	100%

All bacteria were tested for susceptibility to antibiotics using the disk method. Most of these bacteria were highly resistant to all antibiotics. When some susceptibility to an antibiotic was evident which did not inhibit the growth of tubercle bacilli, it was added to the neutralized sediment before inoculating on solid media. This did not prove to be very effective in controlling the contamination even though large amounts were added.

Trisodium phosphate (10%) plus zephiran (1:1000) was effective in controlling contaminants. However, this digestant proved to be toxic to the growth of most tubercle bacilli. Controls were run to determine which strains would grow in spite of this digestion method. This method is not recommended when minimal positive cultures (1-5 colonies) are suspected or for difficult to grow isoniazid-resistant strains.

The amount of trisodium phosphate and zephiran were reduced in

order to minimize the toxicity of this digestion method. These new solutions did not prove to be bacteriostatic to the contaminants in digested, neutralized sputum. In contrast, these solutions did inhibit the growth of pure strains in BHI broth.

Other digestant methods without detergents were sometimes effective such as Darzin's S-P technique[5] and 10% Trisodium phosphate (50 minute exposure). These methods were not used routinely because controlled studies proved the 3% NaOH method generally superior in obtaining growth.

More work must be done to control contamination. The above methods are only applicable in a limited number of specimens.

It was found that the identification of all "contaminants" was worthwhile. Many times, this was the only specimen received and the etiological agent causing disease was "accidently" cultured. For example, 5 cultures of Nocardia asteroides were recovered which had not been sent to the Mycology Laboratory.

Disease causing atypical acid-fast bacilli, especially the fast growing mucoid strains, may be mistaken for a Gram negative rod. How many strains of Mycobacterium fortuitum have been reported as "Contaminated" and autoclaved?

Smear Morphology

A false emphasis has been placed on a smear being merely positive or negative. After several years of observation, we find that there are transitory stages. When the Ziehl-Neelsen Method is used, typical tubercle bacilli stain well. The metachromatic granules which are thought to be metaphosphates stain blue-red. When a patient has been on several months of isoniazid therapy, we see bacilli which are faintly staining and wider. The appearance seems to be that of "toxic swelling". Very few if any granules are seen; instead, we see empty spaces or vacuoles.

The vacuolated faintly staining swollen bacillus does not grow on culture media after prolonged incubation. It is thought to be "nonviable" and an experienced microscopist can predict the outcome of culture growth when adequate sputum specimens are examined over a long period of time.

In 5 cases striking morphology comparisons were made between the patient's smear "positive", culture negative sputums before surgery and the smear "positive" culture negative lung tissue. The bacilli in all specimens had the appearance of "nonviable" bacilli.

Typical
Tubercle Bacillus

"Nonviable"
Tubercle Bacillus

New Positives

Today, many of our new cases of tuberculosis are found in the negative concentrated smear, culture positive group. During the year 1958, 48 of our 83 newly reported positive cultures for tubercle bacilli were minimal positives. Only 1 to 10 colonies grew after 4 to 5 weeks incubation on one out of 3 sputum specimens sent to the laboratory. Sometimes the validity of such a positive culture is difficult to accept because there is no x-ray evidence and very little clinical evidence to substantiate a diagnosis of tuberculosis.

According to Cruickshank's 1952 study,[5] we need 100 tubercle bacilli/ml. of sputum to obtain growth of 1 to 10 colonies. We suspect that many more bacilli are needed when gastrics or urines are cultured. If the tubercle bacilli are exposed to harsh digestants and other adverse conditions, we will have many false negative cultures. We wonder if there are any "false positive" cultures because our culture methods and media are far from perfect.

Atypical Acid-fast Bacilli

Runyon's work on the classification of atypical acid-fast bacilli into the photochromogenic, scotochromogenic, Battey and rapid growing group has been very helpful. We have found that biochemical, colony and smear morphology, and animal virulence studies have been disappointing in determining which strains are capable of causing disease in man.[7] Lung tissue culture of the same organism isolated from sputums or gastrics before surgery is still the best evidence we have as to which atypical acid-fast bacilli cause disease.

It is unfortunate that in the past these organisms have been called Nocardia and tubercle bacilli.[2, 18] Just a few short years ago, those of us who worked in tuberculosis bacteriology laboratories were "instructed" to ignore all atypical acid-fast cultures because they were probably saprophytes. The problem was "solved" when the cultures were autoclaved! Today, TB bacteriologists are saving the cultures, but they don't know what to do with them!

Thirteen hundred atypical acid-fast bacilli cultured from sputums, gastrics, urines, transudates, exudates and tissues and 8 known saprophytes have been studied in this laboratory. All insignificant atypicals and those causing disease were high in catalase activity.[6] They were usually cytochemical neutral red negative[17] and peroxidase negative.[13, 15] Cord formation was usually weak or negative.

The atypical acid-fast bacilli cultures ranged in color from pale buff to bright red. The growth on Lowenstein-Jensen media was compared with Munsell color charts. (National Bureau of Standards Circular 553, 1955). Nothing new could be concluded except to point out that Mycobacterium smegmatis can also be slightly photochromogenic. It might be confused with the disease causing photochromogens. After exposure to light, Mycobacterium smegmatis is "pink" (Munsell color 5.0 YR 7/6) and the photochromogens are "orange" (Munsell color 2.5 Y 8/8). See charts #I, III and V.

(I) SOME CHARACTERISTICS OF 8 PATHOGENIC PHOTOCHROMOGENS

Source	Color		Cord** Formation	Cyto-Chemical Neutral Red ***	Drug Resistance			Catalase Activity	Peroxidase Activity
	Dark	Light			SM ****	PAS	INH		
1. D. M. lung	2.5 Y 8/3	2.5 Y 8/8	2 plus	0	S	R	MR	5 plus	0
2. C. B. lung	2.5 Y 8/3	2.5 Y 8/8	1 plus	0	MS	R	MR	5 plus	0
3. S. S. lung	2.5 Y 8/3	2.5 Y 8/8	+	0	MS	R	R	5 plus	0
4. L. G. lung	2.5 Y 8/3	2.5 Y 8/8	–	0	MS	R	R	5 plus	0
5. S.E. lung	2.5 Y 8/3	2.5 Y 8/8	2 plus	0	MS	R	MR	5 plus	0
6. A. K. lung	2.5 Y 8/3	2.5 Y 8/8	1 plus	0	MR	R	MR	5 plus	0
7. R. F. lung	2.5 Y 8/3	2.5 Y 8/8	2 plus	0	R	R	MR	5 plus	0
8. Kansas City Bacillus	2.5 Y 8/3	2.5 Y 8/8	2 plus	0	R	R	R	5 plus	0

* Munsell color scale.
** Yegian method.
*** Middlebrook Method.
**** Susceptible—S, Moderately susceptible—MS, Moderately resistant—MR, Resistant—R.

(II) ANIMAL VIRULENCE OF 8 PATHOGENIC PHOTOCHROMOGENS

Source	Guinea Pig Autopsied 6 Weeks		Mice .2 mg. IV 3 Weeks	Chicken 1 mg. IV 4 Weeks	Rabbit .1 mg. IV 4 Weeks
	1 mg. IM	1 mg. IP			
1. D. H. lung	1+→0	3 plus	3 plus	3 plus	2 plus
2. C. B. lung	0	3 plus	3 plus	2 plus	0
3. S. S. lung	0	3 plus	3 plus	3 plus	0
4. L. G. lung	0	3 plus	3 plus	3 plus	2 plus
5. S. E. lung	0	3 plus	2 plus	3 plus	0
6. A. K. lung	3+→0	3 plus	2 plus	2 plus	0
7. R. F. lung	0	3 plus	3 plus	3 plus	2 plus
8. Kansas City Bacillus	0	3 plus	3 plus	3 plus	2 plus

(III) SOME CHARACTERISTICS OF SCOTOCHROMOGENS

Source	Munsell Color		Cord Formation	Cyto-Chemical Neutral Red	Drug Resistance			Catalase Activity	Peroxidase Activity
	Dark	Light			SM	PAS	INH		
Pathogens:									
1 M. H. 10 sputums and lung	10.0 YR 7/10	Same	0	±	S	R	R	5 plus	0
2 I. H. sputums	10.0 YR 7/10	Same	0	±	MR	R	R	5 plus	0
3 W. J. Cervical node	10.0 YR 7/10	Same	0	±	S	R	R	5 plus	0
Nonpathogens:									
1 Saprophyte No 4	2.5 YR 7/6	Same	0	±	MS	R	R	5 plus	0
2 Mycobacterium smegmatis	10.0 YR 8/4	5.0 YR 7/6	0	±	MS	R	R	5 plus	0
3 N. G. 2 gastrics	10.0 YR 7/10	Same	0	±	MR	R	MR	5 plus	0
4 A. W. 3 gastrics	10.0 YR 7/10	Same	0	±	MR	R	MR	5 plus	0
5 J. J. Gastric	10.0 YR 7/10	Same	0	±	S	R	R	5 plus	0

Guinea pigs[12] inoculated with 1 mg. intramuscularly and autopsied at 6 weeks were grossly negative and smear negative. Guinea pigs inoculated with 1 mg. intraperitoneally and autopsied at 6 weeks showed some evidence of disease in the livers and spleens and were smear positive. The gross liver lesions were characteristic of atypicals in that they were white, raised, glistening patches. More lesions were evident when disease causing atypicals, especially the photochromogens, were inoculated in this manner.

Mice were grossly positive and smear positive when inoculated with .2 mg. intravenously in the tail vein and autopsied at 3 weeks. The lungs and livers were frequently involved. Spleens were positive when disease causing atypicals were inoculated. Again, the *degree* of virulence was greater when disease causing atypical acid-fast bacilli were inoculated.

Rabbit virulence was variable when inoculated with .1 mg. intravenously in the ear vein and autopsied at 4 weeks.

Chicken virulence was consistent in that 1 mg. of the disease causing atypical acid-fast bacilli inoculated intravenously in the wing vein produced white, raised, glistening patches in the liver and spleen within 4 weeks. These lesions regressed until there was no evidence of disease at 12 weeks. This is in contrast to Avian strains which are inoculated in chickens. The gross lesions are similar to tubercles of Mycobacterium tuberculosis and the disease is progressive in that the chickens usually expire within 12 weeks. See charts # II, IV, and VI.

Some insight was gained by smear morphology of direct sputum specimens and animal tissue. Smears of Battey and Avian strains were very similar. Photochromogens sometimes displayed the large "tygroid" appearance growing in star formation. The smears of one disease causing

(IV) ANIMAL VIRULENCE OF SCOTOCHROMOGENS

Source	Guinea Pig Autopsied 6 Weeks		Mice .2 mg. IV 3 Weeks	Chicken 1 mg. IV 4 Weeks	Rabbit .1 mg. IV 4 Weeks
	1 mg. IM	1 mg. IP			
Pathogens:					
1. M.H. 10 sputums and lung	0	2 plus	2 plus	2 plus	0
2. I. H. sputums	0	2 plus	3 plus	2 plus	1 plus
3. W. I. Cervical node	0	2 plus	3 plus	2 plus	1 plus
Monpathogens:					
1. Saprophyte #4	0	1 plus	2 plus	0	0
2. Mycobacterium smegmatis	0	1 plus	2 plus	0	0
3. N. G. 2 gastrics	0	1 plus	3 plus	0	0
4. A. W. 3 gastrics	0	1 plus	3 plus	0	0
5. J. J. gastric	0	1 plus	3 plus	0	0

V. SOME CHARACTERISTICS OF BATTEY TYPE

Source	Munsell Color	Cord Formation	Cyto-Chemical Natural Red	Drug Resistance			Catalase Activity	Peroxidase Activity
				SM	PAS	INH		
Pathogens:								
1. E.N. 6 sputums and lung	2.5 Y 9/4	±	0	S	R	R	4 plus	0
2. L.A. 2 sputums and lung	2.5 Y 9/4	±	0	S	R	R	4 plus	0
3. Nocardia intracellularis	2.5 Y 9/4	±	0	S	R	R	4 plus	0
Avian:								
1. Kirschberg	2.5 Y 8/3	±	0	S	R	R	4 plus	0
Nonpathogens:								
1. G.M. 3 sputums	2.5 Y 9/2	0	0	R	R	MR	5 plus	0
2. J.F. 1 sputum	2.5 Y 9/3	0	0	S	R	R	4 plus	0
3. F.F. 1 sputum	2.5 Y 9/3	0	0	S	R	R	5 plus	0
4. A.G. 1 sputum	2.5 Y 9/8	0	0	S	MR	R	4 plus	0

Scotochromogen (M.H. on the chart) stained consistently small, almost coccoid acid-fast bacilli. Smears of the saprophytes and insignificant atypicals were indistinguishable from tubercle bacilli on smear.

Nocardia intracellularis[4] (culture obtained from Dr. Conant, Duke University) appeared to have all of the characteristics of the Battey strains and is included on charts #V and VI. No branching was evident on smears of animal tissue. Color, virulence studies, catalase and peroxidase activity proved it to be identical to our disease causing Battey atypicals.

VI. ANIMAL VIRULENCE OF BATTEY TYPE

Source	Guinea Pig Autopsied 6 Weeks		Mice .2 mg. IV 3 Weeks	Chicken 1 mg. IV 4 Weeks
	1 mg. IM	1 mg. IP		
Pathogens:				
1. E. N. 6 sputums and lung	0	3 plus	3 plus	2 plus
2. L.A. 2 sputums and lung	0	3 plus	3 plus	2 plus
3. Nocardia intracellularis	0	1 plus	3 plus	2 plus
Avian:				
1. Kirschberg	0	3 plus	3 plus	3 plus
Nonpathogens:				
1. G.M. 3 sputums	0	1 plus	2 plus	0
2. J.F. 1 sputum	0	1 plus	1 plus	0
3. F.F. 1 sputum	0	2 plus	2 plus	0
4. A.G. 1 sputum	0	2 plus	2 plus	0

Discussion

Some of the confusion surrounding the disease causing atypical acid-fast bacilli could be lessened if definite names were assigned such as Buhler and Pollack's[1] photochromogen, "Mycobacterium Kansasii". The pathogenic atypicals deserve a prominent place in the classification of bacteria.

Patients from whom atypical organisms have been cultured are placed on the tuberculosis service even though we have no evidence that these atypicals are as infectious as Mycobacterium tuberculosis. Clinically and surgically, many patients do not respond to antituberculous therapy. Therefore, the diagnosis of tuberculosis doesn't seem feasible in the absence of the tubercle bacillus.

Slowly, we are finding explanations for the smear positive, culture negative phenomena. This is in spite of crude culture methods and media. Many references have been made to inadequate tissue culture techniques. Are we sure that these bacilli are viable? This leads us to the question of whether or not surgery should be performed on some patients. Is the need for surgery indicated when these bacilli which appear to be nonviable are seen? Instead, this might mean that the patient should have a longer course of chemotherapy.

There are other clinical implications associated with the reporting of vacuolated, faintly staining, swollen bacilli which appear to be nonviable.

1) Many patients have become smear and culture negative and are placed on the "negative" end of the ward. After several months, a few "nonviable" bacilli may be found. In light of these findings

together with clinical and x-ray evidence, it is not feasible to place the patient on the "positive" end of the ward. These bacilli are usually transient and the patient usually is completely smear and culture negative within a few weeks.

2) Unfortunately, these bacilli are sometimes found when a patient is ready for discharge. The correct emphasis on their importance should be considered.
3) A decision for surgery to initiate a complete cure because of "slow conversion" on the basis of these bacilli would seem to be a false conclusion.

Those of us who work in tuberculosis bacteriology laboratories have the opportunity to observe the typical susceptible tubercle bacillus become an isoniazid-resistant variant which is entirely different from Robert Koch's bacillus. We can not expect the isoniazid-resistant variant to conform to Koch's standards of smear and colony morphology, virulence, etc. This is a variant and has standards of its own. The cytochrome enzyme system is altered and the bacillus may grow only on special media in CO_2. The fate of this variant is unknown. Is it capable of producing disease in another human being? If this occurs, is the infected patient's tubercle bacillus typical or an isoniazid-resistant variant?

The bacteriology of tuberculosis is a challenge! The tubercle bacillus has proven itself to be a very versatile organism. We medical technologists and bacteriologists must learn to be as versatile in adopting new methods and standards.

There is also a need for those who work in the laboratory to communicate the phenomena they have observed in the bacteriology of tuberculosis. The clinicians and bacteriologists can only hope to explain these phenomena by working together and keeping thorough patient records. When this is done, there will be renewed interest in treating the tuberculosis patient.

Summary

Each specimen must be treated in a different manner because each patient's tubercle bacillus has different growth requirements. These may vary in the course of treatment. Within the limitation of tuberculosis laboratory personnel, space and money, optimum results will be achieved when:

1) More than one digestant method is used.
2) More than one type of media is used.
3) CO_2 is added for growth of isoniazid-resistant catalese deficient variants.
4) Cultures are incubated for 3-6 months.

Two types of isoniazid-resistant tubercle bacilli have been observed. A microscopist who is experienced in the morphology of the tubercle bacillus should look for *all* the vacuolated, faintly staining, swollen bacilli characteristics in direct sputum and tissue smears. These bacilli are thought to be *nonviable,* in the absence of typical staining tubercle bacilli, and can explain a negative culture.

The other type of isoniazid-resistant tubercle bacilli are *viable* and stain well. They are very acid-fast and cell morphology is easily seen. The

bacilli grow only in the presence of or better in 10% CO_2. The colonies may be flat and smooth. Catalase activity is negative to 3 plus. Peroxidase activity is usually absent.

Atypical acid-fast bacilli virulence studies can be misleading. Care must be taken to standardize the inoculum, route of inoculation and time of necropsy. Chickens inoculated intravenously with 1 mg. of a disease causing atypical culture and autopsied at 4 weeks showed consistent liver and spleen lesions. In the absence of typical tubercle bacilli, the same atypical organism should be cultured from sputums or gastrics before surgery and from the lung tissue.

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A NEW ATTACK ON TB*

The program recently announced by the National Tuberculosis Association to wipe out tuberculosis in the United States by locating every person suffering from the disease and treating him with appropriate medication should soon add still another one-time killer to the long list of drug-eliminated ailments.

As in the case of many other one-time killers, the Health News Institute points out, new medicines have made it possible for the medical profession to not only cut the death rate from tuberculosis, but to hope for its complete eradication as well. Since 1940, new advances in medicine have helped cut the death rate from tuberculosis from 45.9 of every 100,000 Americans to only 7 per 100,000 in 1958.

The steep drop in less than twenty years is due primarily to the development of streptomycin by Dr. Selman Waksman of Rutgers University (backed by a substantial grant from a large pharmaceutical manufacturer); isoniazid, developed specifically for use in tuberculosis by American pharmaceutical manufacturers, and PAS (paraaminosalicylic acid). These are the three drugs with which the National Tuberculosis Association plans to treat every tuberculosis sufferer in the United States, and thus eradicate the disease for all time.

But tuberculosis is only one of many diseases which have responded in a similar fashion to new medicines. Such once dreaded diseases as typhoid fever, diphtheria, mastoiditis, rheumatic fever, poliomyelitis, the venereal diseases and many others have lost their sting. Pneumonia and influenza take less than half the number of lives they took in 1940. Maternal deaths have dropped from almost 40 per 10,000 live births in 1940 to 4 per 10,000 in 1958.

As these diseases have been defeated by new medicines, others have come to take their place. But our hope for the future, the HNI says, is that some day soon, the American Cancer Society, the American Heart Association, or the National Committee Against Mental Illness may be able to report that it hopes to eradicate these diseases by tracking down every victim of their ravages and treating them with such widely available products of the pharmaceutical industry as streptomycin, isoniazid and PAS.

* From Health News Institute, 60 East 42nd St., N. Y., N. Y. March 1960.

NORMAL RANGES IN CLINICAL WORK*

Their Uses and Methods of Determination

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The term "normal values" as used in clinical work refers to the amount of a constituent present in the body fluid or excretion of a healthy human being. These values are not constant, but may vary over a range. In fact, even individuals, seemingly normal in every respect, may have values outside the "normal ranges" for certain constituents. An interesting experiment was done by Williams, Brown, and Shideler (Williams, et al, 1955)[8] on eight male medical students. They did a set of 19 tests on each and found that though these men were apparently free of any systemic disorders, they gave an "abnormal" result on at least one of the tests in the set.

Detection of a truly diseased patient, therefore, does not depend on finding one abnormal biochemical result. It depends on the total data available, the experience at the clinician's command and on an accurate knowledge of the normal from which to interpret results. In expressing the normal values for a constituent, a narrow range will exclude many normal people. Enlarging this range will include more normals, but "a range that tries to include all possible normals would be uselessly large (King and Wootton, 1953).[6]" Therefore, in using any range for diagnostic purposes, a knowledge of how this range was obtained is important.

Normal limits serve two fairly distinct functions. These are description and discrimination.[5] The medical technologist is interested primarily in the descriptive function, while the clinician is concerned with the second aspect because it aids in discriminaton of healthy and diseased individuals. In this sense, normal limits act in a large capacity as a screening device, ruling out certain possibilities with relatively high probability. The smaller the overlap between the distribution of values in healthy and in diseased persons, the more useful the range becomes. To place correct value on the usefulness of specific normal ranges for diagnostic purposes, a comparison should be made by examining ranges of variation for the same constituents in disease. Very little has been done in this line as yet.[5] In most cases, therefore, the clinician has to rely on the ranges of normals alone.

Probably the two most difficult problems in establishing the normal ranges are 1) the problems concerned with definition as discussed above and 2) the selection of subjects. For the second, a large group is needed composed of subjects who can be considered typically healthy as based on criteria other than the measurement being considered. People commonly considered "normal" appear healthy and free from any disability or disease which might be expected to affect the measurement in question. For some measurements, "the normal limits even for healthy persons varies with age, sex, race, or diet, altitude, climate and other environmental conditions (Austin, 1949)[2]."

Recognizing this, E. J. King set up his chart on normal values for 27

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* Claudia M. Stannard Award 1959. Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959.

different blood constituents,[7] and used 50-100 analyses for each set of values calculated. His subjects were of both sexes and ranged in age from 18 to 50. Upon examination, they were found free of any systemic diseases. Previous to obtaining the samples to be analyzed, the subjects fasted for at least four hours.

Once the data has been collected, the next step in establishing the normal range is to select a method of determining the outside limits of variation for constituents in "normal" humans.

Several methods are now in use. The most common of these is statistical computation of the mean and standard deviation of available data. The outside limits are set at 2 standard deviations.[3] This results in a "95% normal range" in which only 5% of normal subjects fall outside of the limits.

Though this would seem to be the most convenient way to express the normal limits, it gives reliable values only when two conditions are fulfilled.

First, the mean and standard deviation must be calculated from very large samples so that they may be considered known quantities.[5] However, too frequently the sample is too small to assure an accurate cross section of the normal, and could result in an inaccurate normal range. This diminishes the reliability of the method.

Second, the measurements must form the mathematical distribution called the Gaussian and often, misleadingly, the "Normal" distribution. See figure 1. (Gauss, Leplace, Maxwell, and others were the first to develop the bell curve as a useful mathematical tool.)

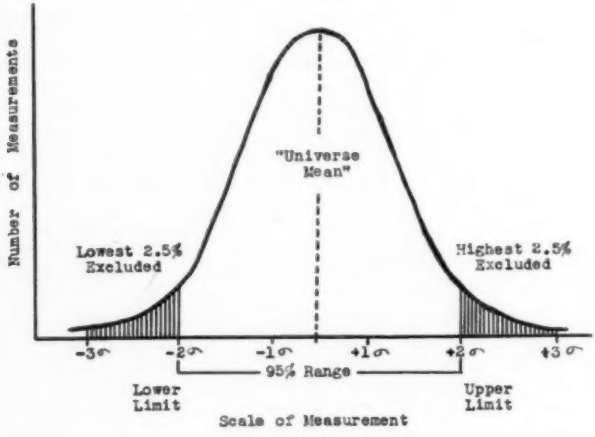

Figure 1
A "Normal" distribution

Again the sample must be fairly large to assure a Gaussian distribution. To assume that biological measurements usually have this distribution would be both unwarranted and dangerous and a small sample would

require this assumption. Wootton, King, and Smith[6] have shown that only about one-half of blood constituents tested gave "Normal" distributions while the rest were all positively skewed. The only exception was plasma globulin, which was negatively skewed. If normal ranges were established with this method from a skewed distribution, serious misclassification of cases could have resulted depending upon the degree of skewness.

Another common method for determining normal limits is simply to use the highest and lowest values obtained in a sample of measurements. This is obviously a much less accurate method, and should be used in preference to others only when the sample size is too small to be fitted to a frequency curve and the values show convincing evidence that they are asymmetrical in distribution.

Despite the fact that the above two methods of obtaining normal limits are most commonly used today, the most accurate method for obtaining the 95% range is by comparison of the plotted frequency distribution to a Gaussian curve (Albritton, 1952)[1], Figure 2. If the two do not correspond, that is, if the plotted distribution is skewed, then comparison is made between the plotted distribution and the lognormal curve, as in Figure 3.

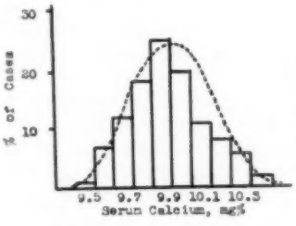

Figure 2
Comparison with a normal distribution.

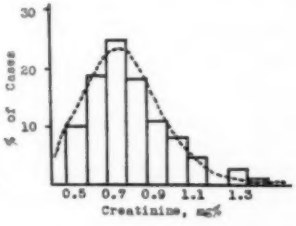

Figure 3
Comparison with a skewed distribution.

The lognormal curve is derived by transforming the distribution by means of some function of the actual observations so that the original distribution becomes normal. If this function is used instead of the observations themselves, subsequent calculations are simplified and their scope is extended. Derivation of formula used: If x = skewed distribution and the f(x) = normal distribution, taking f(x) = log x, the formula becomes: Transformation X = log x.[4] This method is often found useful with biological data, and the same procedure as for normal distributions is followed to obtain the normal range. The negatively skewed distribution is fitted with limits by inspection.[9]

Differing from the above methods based upon standard deviation is the percentile method of determining normal limits. It isn't new, being first proposed by Thompson and later more exhaustively described by S. S. Wilks[5] in 1948.

This method has several great advantages. First of all, it can be used with accuracy regardless of the sample size. Secondly, no assumptions

need be made as to the distribution shape (Gaussian or other) of the sample. Those clinical investigators who are convinced that local norms* are important take particular preference to this method.

In using the percentile method to determine the 95% range, we simply find the 2.5 and 97.5 percentiles in the form of the corresponding sample percentiles, thus excluding 2.5% of the sample above the upper limit and 2.5% below the lower limit. For example, to illustrate and describe the 2.5 percentile very simply, let us assume that we have a sample size of 160 values that has been ranked in order from the lowest to the highest, lowest being #1, therefore, item of rank 1. To determine the 2.5 percentile, we multiply 2.5% by 160, which equals 4. Therefore, the 5th item of rank would become the lower limit of normal and the 156th item of rank would become the upper limit of normal. The drawback to the 95% range using percentiles seems to lie in the fact that for screening procedures in which potentially ill people are to be studied more intensively, it seems more preferable to draw a narrower range for acceptance as healthy. This way gives a better chance of detecting abnormal subjects in situations where the overlap between the distribution of values in the healthy and diseased is quite large.

To draw a narrower range, the 10 and 90 percentiles are used. The range here is determined in the same way as for the previous percentiles described, except that the sample size (n) should be chosen so that $(n + 1)$ multiplied by 10% will come out a whole number.** If the estimated percentile comes out, say, 15.6, interpolation between the 15th and 16th items of rank would have to be used and this would bring in some inaccuracy into the estimation. King and Wootton calculated two different normal ranges for 27 blood constituents (See Table I, King and Wootton, 1953)[6] using an inner narrower range that included 80% of normal findings and an outer range which included 98% of normal people. Values falling outside of the narrow range can be considered suspicious of being abnormal, but values falling outside the 98% range can be treated as almost certainly abnormal.

Another set of percentiles used but not so popular is the 5 percentile which is understood to exclude more than 5% of population values, and 15 percentile which excludes less than 15% of population values. It might be desirable to use the exclusion of at least 5% of standard (healthy) population in screening for differential diagnosis when certain symptoms are really present. Although this would exclude a number of healthy people, when the method was applied to a small sample, still the likelihood of a healthy person coming in for clinical diagnosis may be small enough to justify use of this standard. Another time when this 5 percentile might be of value is when the people who make up the average of the population are to be studied in a small sampling. Use of the 15th percentile might be warranted in screening groups

* Local norms, which must often be based on small samples, consist of standards set up in a particular location and in some cases are restricted to people with closely specified characteristics. Really, every norm is a local norm to a greater or lesser degree.

** The use of $(n + 1)$ has been shown to give a better more unbiased estimation of percentiles (Herrera, 1958).[5]

TABLE I
Normal values for blood constituents.
King and Wootton

	Lower 1%	Lower 10%	Higher 10%	Higher 1%	Remarks
Whole Blood:					
Urea, mg%	12	16	35	47	Log normal
NPN, mg%	25	29	43	51	Log normal
Uric acid, mg%	0.6	1.6	3.9	4.9	Normal
Creatinine, mg%	0.1	0.1	1.2	2.6	Log normal
Phosphate (inorg.), mg%	2.0	2.4	3.5	3.9	Normal
Cholesterol, mg%	115	140	215	265	Log normal
Glucose, mg%	55	68	96	109	Normal
Chloride, as NaCl, mg%	425	454	526	555	Normal
Serum or Plasma:					
Na (meq./l.)	133	137	148	152	Normal
K (meq./l.)	3.5	3.9	5.0	5.6	Log normal
Ca (meq./l.)	4.5	4.8	5.4	5.7	Normal
Cl (meq./l.)	99	101	106	108	Normal
CO_2 combining power (meq./l.)	24	25	29	31	Normal
Total protein, gm%	6.1	6.7	7.7	8.2	Normal
Albumin, gm%	4.0	4.4	5.3	5.7	Normal
Globilin, gm%	1.5	1.9	2.8	3.0	Neg. skew
Fibrin, gm%	0.1	0.2	0.4	0.5	Normal
Bilirubin, mg%	0.1	0.1	0.5	0.8	Log normal
Cholesterol, mg%	123	153	260	324	Log normal
Phosphate, inorg., mg%	2.4	2.9	4.1	4.5	Normal
Phosphate total in acid solution, mg%	2.7	3.2	4.3	4.7	Normal
Phosphate, ester, mg%	0.0	0.1	0.6	1.7	Log normal
Phosphate, lipid, mg%	7.0	8.3	12.6	14.9	Log normal
Alkaline p'tase, units	3.3	4.5	9.5	12.9	Log normal
Acid p'tase, units	0.8	1.2	3.1	4.6	Log normal
P'tase formol stable, units	0.0	0.0	2.1	4.1	Log normal
Amylase, units	71	91	163	209	Log normal

consisting mainly of healthy souls, of which the number to be watched should be kept low.

With all these ways of setting up normal ranges, it becomes imperative to state always what percent of normals is being included in the range in order to aid the clinician in judgment of his cases.

At the University Hospitals, Minneapolis, Minnesota, the method for determining normal ranges is the percentile method of estimating the 95% range. This is the best suited since, as previously stated, no attention need be paid to the size or distribution of the sample. In another method occasionally used, a sample consisting of as few as 10 seemingly healthy persons is tested. The experiment is followed exactly as its author sets it up. The results are checked to see if they fall within the normal limits set up by the author for his experiment. If they compare favorably, the normal limits are adopted.

In attempting to check the validity of the normal values as employed in this hospital's chemistry laboratories, this writer gathered 200 values from recent chemistry protocol books, for each of the blood constituents listed in Table II below, and attempted by simply excluding the upper and lower 2.5% (5 values) to establish a range that would approximate normal. By comparing this range with the one routinely used in the lab, I hoped to see how well values currently obtained were correlated to the ranges previously set up. This method proved grossly unsatisfactory since the upper and lower "normal" limits were too widely spread. They were diverse enough to include all normals as well as a large percentage of abnormals. This disproved any ideas one might have had

TABLE II

"Normal Limits" of the Experimenter
(Based on 200 determinations done on patients.)

	Lower 2.5%	Lower 15%	Higher 15%	Higher 2.5%	Range Now in Use
Serum or Plasma:					
Calcium, mg%	6.9	8.3	10.0	10.6	9.5–10.5
Phosphorus, mg%	2.1	2.7	4.8	5.8	2.5–4.0
CO_2 combining power (meq./l.)	16	21	30	34	26–28
Chloride, (meq./l.)	86	95	107	116	100–106
Sodium, (meq./l.)	124	131	143	153	137–147
Potassium (meq./l.)	2.9	3.7	5.4	6.3	4.0–5.5
Total protein, gm%	4.7	5.5	7.3	7.9	6.7–8.4
Albumin, gm%	1.7	2.3	3.8	4.1	3.6–5.6
Globulin, gm%	2.1	2.6	4.0	5.2	2.0–3.7
Creatinine, mg%	0.6	....	2.6*	10.9	0.5–1.5
Acid p'tase, units**	0.9	....	2.0*	7.6	0.0–3.3

* In disease, these two constituents are always increased; therefore, to establish the upper limits of "normal" in the narrower range, 30% was excluded whereas the lower limit remained at 2.5% excluded. In this way, the same number of values was excluded from the original 200 as for the other constituents.
** Only 100 values were used since this determination is not done as frequently as the others used.

concerning the reliability of this method for calculating a useful normal range. In selecting these 200 sample values, care was taken to avoid emergency work, and values appearing extremely abnormal.

For most measurements of normal values in clinical chemistry recorded by J. Harold Austin in his book,[2] they were dependent on the range commonly found in hospital experience in patients whose condition justified no suspicion of abnormality of the measurement in question. In these cases, the ranges selected is rather arbitrarily chosen to include most supposedly normal persons and to exclude most supposedly abnormal persons. These ranges gain acceptance only after they are found to conform to the experience and judgment of other workers. From this information it would appear that if this writer were to use previously obtained values and attempt to get a useful range from them, all the patients used as subjects would have to be screened and those eliminated with diseases which could conceivably alter the value in question.

In this paper, frequency distribution graphs were drawn for the constituents and an interesting feature of proteins showed up here. Total protein content in the blood, it seems, decreases by as much as one gram % in a person who is lying down for long periods of time previous to the testing. For this distribution, the writer got a bimodal curve, the lower peak at 5.5 gm% denoting the mode of patients in bed and the higher peak at 6.8 gm% representing the mode of patients who aren't confined to bed. This representative sample of 200 could not, therefore, be used with accuracy until one eliminated the patients who had been in the prone position for a while previous to the drawing of the test sample. The frequency distribution graphs also showed whether the samples were normal or skewed. In this respect, all samples treated in this way gave a curve corresponding to those previously done by other experimenters with the exception of potassium. The acid phosphatase and creatinine showed positive skewness, but although potassium was also supposed to be postively skewed, it ran a very borderline

and from this 200 sample it was difficult to say if this was a Gaussian distribution or not. The sample size would have to be increased to be sure. It became apparent to the writer that the reason for these skewed curves was that in any systemic disorders of a kind affecting these values in question, the values would be almost always increased by the disease affect and rarely if ever would a decreased value occur. On the other hand, normal distributions resulted when the diseases affected those values, either increasing or decreasing them.

By using the 15 percentile in a slightly different manner from what is described above, the writer tried to narrow the ranges she had determined by eliminating from the normal distributions the upper and lower 15%. The ranges were narrowed appreciably, see Table II, and approached more closely those currently in use in the laboratory. However, there was a tendency for the ranges to be lower. This suggests that either most values are affected somewhat by the prone position of a patient as are the proteins, or that the values obtained daily in the lab actually fall into a different normal range from those commonly accepted. To explain this latter suggestion, we could turn to "local norms." Technologists have observed that in the case of potassium, normal results obtained in this hospital do fall into a range slightly lower than that stated by the author of the experiment.

We have seen that there are numerous ways of establishing normal ranges in hospital laboratory work. Therefore, it becomes necessary to state the percentage of normals included in order that the normal limits will not be misleading in diagnoses. Standard practice applies these procedures for the benefit of the individual patient only. It has been shown, however, when considered from another point of view, a study of collective data of these individual patients can be used for setting up the limits of normal. Hospital laboratories will provide a rich source of this quantitative data. Finally we have seen that more accurate ranges can be obtained with careful selection of the quantified data, that is, by using only those values from patients whose illness does not tend to affect the constituent in question. Thus, the statistical treatment of biochemical results can aid the clinician to ever increasing accuracy in disease diagnosis.

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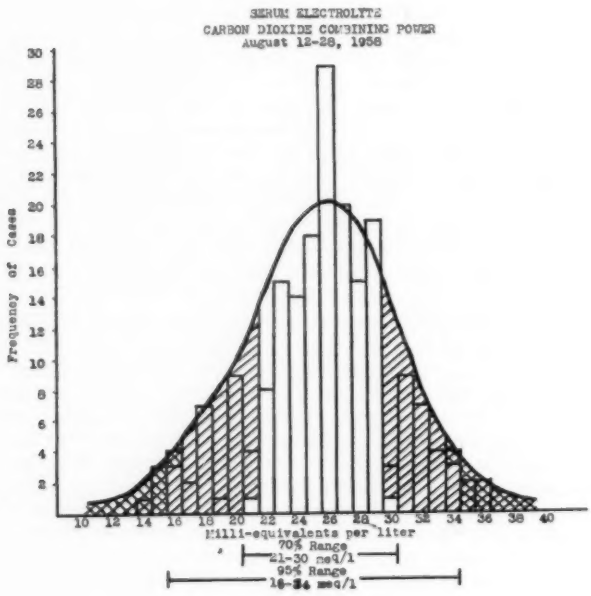
APPENDIX



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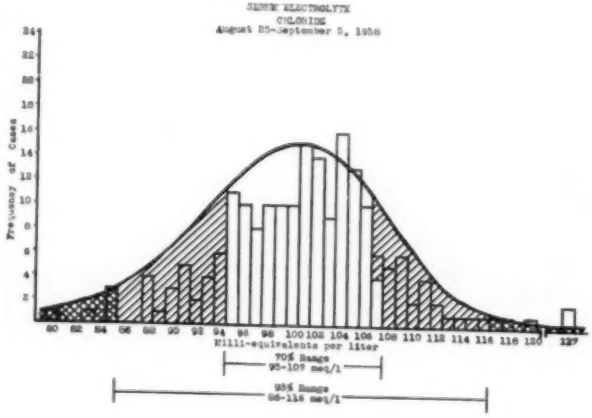

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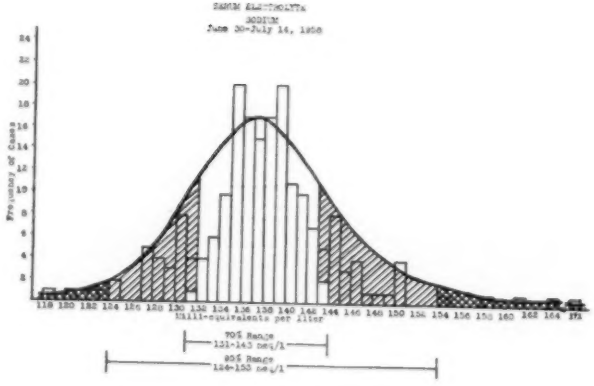

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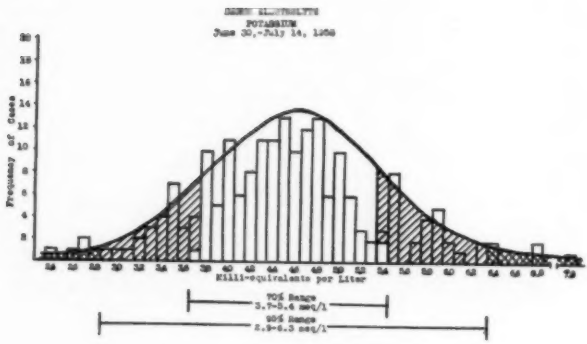

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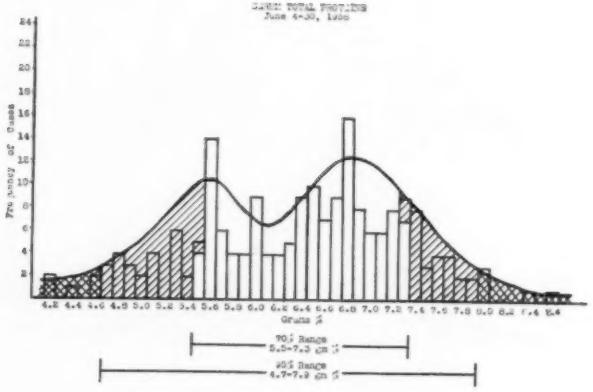

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ABSTRACTS

A "NEW" ANTIBODY, ANTI-Rh^c, RESULTING FROM ISOSENSITIZATION BY PREGNANCY WITH SPECIAL REFERENCE TO THE HEREDITY OF A NEW Rh-Hr AGGLUTINOGEN Rh$^c_2$

Lester J. Unger and Alexander S. Wiener (New York University-Bellevue Medical Center, and Office of the Chief Medical Examiner, Nw York, N. Y.) J. Lab. Clin. Med. 54, 835-42 (1959).

The observation is made that standard Rh$_o$-positive individuals can be isosensitized by Rh$_o$-positive blood and produce antibodies resembling anti-Rh$_o$ in specificity. It is shown that in Rh agglutinogens the Rh$_o$ factor has other blood factors associated with which have been designated Rh^A, Rh^B, Rh^C, Rh^D, etc. Certain rare Rh$_o$-positive individuals may lack one or more of these associated factors and may become sensitized by "standard" Rh-positive blood. The antibody produced is indistinguishable from ordinary anti-Rh$_o$ in specificity except that it fails to react with the Rh$_o$-positive blood of the antibody producing individual as well as other rare Rh$_o$-positive bloods.

The case presented gave birth to fraternal male twins, both Rh$_o$ positive and with signs of mild erythroblastosis fetalis. The antibody in her blood was assigned the symbol anti-Rh^C as it differed from anti-Rh$_o$, anti-Rh^A and anti-Rh^B. A method for preparing ficinated cells and their advised use in detecting sensitivity in cross-matching blood for transfusion is strongly emphasized.

USE OF THE PLASMA THROMBIN TIME TO ASSESS THE ADEQUACY OF IN VIVO NEUTRALIZATION OF HEPARIN: COMPARATIVE STUDIES FOLLOWING OPERATIONS EMPLOYING EXTRACORPOREAL CIRCULATION

James C. Peden, Jr., and James A. McFarland (National Institutes of Health, Bethesda, Md.)

*The increasing frequency of operations employing extracorporeal circulation with its addition in many cases of 1.5 mg. of heparin per Kg. of body weight and the addition of 40 mg. of heparin per liter in the blood used to prime the pump-oxygenator has led post-operatively to the immediate slow intravenous injection in about 50 ml. of 5% glucose of 1-1.5 mg. of protamine for each mg. of heparin calculated to be present.

When complete mixing of the administered protamine was judged a blood sample was drawn for thrombin time and protamine titration. If either were abnormal more protamine was given until both tests indicated complete neutralization of heparin. The plasma thrombin time was far more sensitive in detecting small quantities of residual heparin. Subsequent frequent determinations were made for prothrombin time and silicone and Lee-White clotting times during the first 12 hours post-operatively. The study was made on 25 patients.

* Blood XIV, 1230-36 (1959).

5-HYDROXYTRYPTOPHAN AS AN INTERMEDIATE OF SEROTONIN BIOSYNTHESES IN MALIGNANT CARCINOIDOSIS

Robert M. Donaldson, Jr. et al (Peter Bent Brigham Hospital, Boston) Lancet II, 1002-3 (1959).

Reference to the studies of Udenfriend et al (1956) and Davidson et al (1957) which suggest that in the body the first step in serotonin formation is the hydroxylation of tryptophan to form 5-hydroxytryptophan.

Urine from 5 patients with malignant carcinoidosis was studied. 5-hydroxyindole compounds were identified. In 3 of these the urine contained only a single nitroso-naphthol-reacting compound with chromatographic properties similar to 5-hydroxy-indoleacetic acid. In the other 2, three 5-hydroxyindole compounds were demonstrated.

The presence of 5-hydroxytryptophan in the urine of 2 patients with malignant carcinoidosis and the conversion of C-tryptophan to 5-hydroxytryptophan-C in vivo lend further support to the concept that 5-hydroxytryptophan is an intermediate in the biosynthesis of serotonin.

THE SEROLOGY AND THE PROGNOSIS OF 128 CASES OF AUTOIMMUNE HEMOLYTIC ANEMIA

Jean Dausset and Jacques Colombani (Centre National de Transfusion Sanguine, Paris) Blood XIV, 1280-1301 (1959).

The anemic patients were selected by two criteria-a positive direct Coombs test or a cold agglutinin titer exceeding 1-32. Such cases were further divided into "idiopathic" and "symptomatic" varieties, and warm and cold antibodies. Thus five principal forms of the disease were distinguished with their treatment and prognosis. In all cases of cold antibodies exposure to cold had to be avoided; in cases of hemolysins, washed red cells had to be used for transfusions.

EFFECTS OF AN ACUTE SALT LOAD IN A CASE OF PRIMARY HYPERALDOSTERONISM BEFORE AND NINE MONTHS AFTER SURGICAL CURE. Comparison With Normal Subjects And Patients With Essential Hypertension Before And After Reduction Of Their Elevated Pressure With Drug Treatment.

Ramon Ortuzar et al (Catholic University of Chile, Santiago) J. Lab. Clin. Med. 54, 712-21 (1959).

The effects of an injection of hypertonic saline solution before and nine months after the surgical removal of an adrenal adenoma are compared with those observed with the same test on normal subjects and patients with essential hypertension.

Before surgery in the case of primary hyperaldosteronism and that of essential hypertension gave the same response to the sodium loading. After surgical cure the patient with primary hyperaldosteronism gave a response similar to that obtained in drug-reduced hypertensive patients.

A mechanical pressure factor acting upon glomerular filtration more than a hormonal factor of cortical type acting on tubular reabsorption accounts for the increased excretion of water, sodium and chloride produced by a hypertonic saline injection in such cases.

PROTEIN FRACTIONATION

I. CHROMATOGRAPHY OF HUMAN SERUM PROTEINS ON D E A E-CELLULOSE WITH SPECIAL REFERENCE TO BLOOD GROUP ANTIBODIES

R. J. Speer et al (Baylor University, Dallas) J. Lab. Clin. Med. 54, 685-93 (1959).

D E A E-Cellulose (2-diethyl-amino-ethyl ether) as an anion exchanger was utilized for resolving complex protein mixtures with several different buffer systems. The fractionization of antibodies reacting with human erythrocytes (anti-A, and anti-A_1, anti-B and anti-D) and the separation of cryptagglutinoid from agglutinin was accomplished. Radioactively labeled proteins aided the evaluation of resolution.

II. CHROMATOGRAPHY OF SERUM PROTEINS OF PATIENTS WITH ACUTE LEUKEMIA AND MULTIPLE MYELOMA

Morton D. Prager et al (Baylor University, Dallas) J. Lab. Clin. Med. 54, 694-706 (1959).

Normal serum and a pathologic serum (patients with leukemia or multiple myeloma) were studied simultaneously on two columns prepared under identical conditions and eluted by buffer flowing from a single mixing chamber and results were reported. The serum protein changes in leukemia did not compare in magnitude with those observed in multiple myeloma. Proteins from multiple myeloma patients were further differentiated chromatographically.

THE USE OF SIMPLE MATHEMATICS IN ANSWERING CERTAIN LABORATORY QUESTIONS*

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Introduction:

Often people new to our laboratory, such as summer workers or recent college graduates, have asked questions for which I have had to find simple answers. Presented here are some of the more frequently asked of these questions which can be answered by the use of simple mathematics.

Questions and Answers:

Question: Why does a sugar solution, 2 ml. of which contain 0.4 mg. of glucose, represent a 200 mg./100 ml. standard?

Given: In this particular blood sugar determination, we use 2 ml. of standard solution and 2 ml. of whole blood protein-free filtrate (2 ml. filtrate = 0.2 ml. of whole blood).

Answer: We have to relate the three different fluids: 2 ml. of standard solution, 2 ml. of whole blood protein-free filtrate, and 100 ml. whole blood.

(1) 2 ml. of standard = 0.4 mg. of glucose.
(2) Relate 2 ml. of standard to 1 ml. of blood by multiplying 0.4 by 5, since 2 ml. of blood filtrate = 0.2 ml. or 1/5 of 1 ml. of blood.
(3) To relate 1 ml. of blood to mg./100 ml. we multiply by 100.

Hence: $0.4 \times 5 \times 100 =$ *200 mg./100 ml.*

Question: Why does a blood urea nitrogen solution, 2 ml. of which contains 0.03 mg. of urea, represent a 15 mg./100 ml. standard?

Given: In the determination we use 2 ml. of standard solution and 2 ml. of whole blood protein-free filtrate (2 ml. filtrate = 0.2 ml. of whole blood).

Answer:

(1) 2 ml. of standard = 0.03 mg. of urea.
(2) Relate 2 ml. of standard to 1 ml. of blood by multiplying 0.03 by 5, since 2 ml. of blood filtrate = 0.2 ml. or 1/5 of 1 ml. of blood.
(3) To relate 1 ml. of blood to mg./100 we multiply by 100.

Hence: $0.03 \times 5 \times 100 =$ *15 mg./100 ml.*

Question: Why does a cholesterol solution, 2 ml. of which contains 0.8 mg. of cholesterol, represent a 200 mg./ 100 ml. standard?

Given: In the determination we use 2 ml. of standard solution and 0.4 ml. of serum for the unknown.

Answer:

(1) 2 ml. of standard = 0.8 mg. of cholesterol.
(2) Relate 2 ml. of standard to 2 ml. of serum by multiplying 0.8 by 5, since 0.4 ml. of serum = 1/5 of 2 ml. of serum.
(3) To relate 2 ml. of serum to 100 ml. of serum we multiply by 50.

Hence: $0.8 \times 5 \times 50 =$ *200 mg./100 ml.*

Question: Why does a $NaHCO_3$ solution, 1 ml. of which contains 0.025 mM., represent a 25 mM/L. standard?

* Received for publication Feb. 1960.

Given: In the determination we use 1 ml. of standard solution and 1 ml. of plasma.

Answer:

(1) 1 ml. of standard = 0.025 mM of $NaHCO_3$.
(2) Relate 1 ml. of standard to 1 ml. of plasma by multiplying 0.025 by 1.
(3) To relate 1 ml. of plasma to 1 liter we multiply by 1000.

Hence: $0.025 \times 1.0 \times 1000 = 25$ mM/L.

Question:

(1) Why are eight volumes of N/12 sulfuric acid and 1 volume of 10% sodium tungstate used in making a whole blood protein-free filtrate?
(2) Why does the above combination of solutions produce an isoelectric solution?

Answer:

(1) We read[2] that complete protein precipitation takes place only at the isoelectric point. A combination of eight volumes of N/12 sulfuric acid and one volume of 10% sodium tungstate produces an isoelectric solution.
(2) (a) N/12 sulfuric acid = 1/12 = .0833 Normal.
(b) 10% sodium tungstate gives a solution, 100 ml. of which contains 10 gms. of $Na_2WO_4 \cdot H_2O$.
$Na_2WO_4 \cdot 2H_2O = 2(23) + 184 + 4(16) \cdot 2[2(1) + 16] = 330.$
$Na_2WO_4 = 2(23) + 184 + 4(16) = 294.$
$2H_2O = 2[2(1) + 16] = 36.$
The percentage of water of crystallization would be

$$\frac{36}{330} \times 100 = 10.90909 = 10.91\%.$$

The percentage of Na_2WO_4 would be $\frac{294}{330} \times 100 = 89.0909 = 89.09\%$.
Since Na_2WO_4 is 89.09%, 100 ml. of a 10% solution would contain 8.909 gms. and 1000 ml. would contain 89.09 gms.
A N Na_2WO_4 solution would be ½ molar or $\frac{294}{2} = 147$ gms./1000 ml.
Hence the sodium tungstate solution is $\frac{89.09}{147} = .6061$ normal.
(c) 8 ml. $\times$ .08 N H_2SO_4 = .6
1 ml. $\times$ .6 N Na_2WO_4 = .6 = Isoelectric point

Question: If, in a liver function test, 5 mg. of dye is given per kilogram body weight, how many ml. of dye solution are given for a 100 lb. patient if each ml. of dye solution contains 50 mg. of dye?

Answer:

(1) To convert 100 lb. to grams, we multiply by 454 (number of grams in a pound).
(2) Since there are 1000 grams in a kilogram, we divide the body weight in grams by 1000 to obtain the body weight in kilograms.

(3) Then we multiply kilograms of body weight by 5 to obtain mg. of dye dosage per kilogram of body weight.
(4) Finally we divide by 50 to determine ml. of dye solution to use.

Hence: $\frac{\left(\frac{100 \times 454}{1000}\right)5}{50} = 4.54 = \textit{4.5 ml. of dye given.}$

Question: If a certain commercially prepared serum base solution having a listed value of 595 mg./100 ml. chloride as NaCl, and a normal range for the method used listed as 630-580, what approximate value could one expect to obtain from this solution if it were determined by the method used in one's own laboratory which lists a normal range of 630-560?

Answer:
(1) Value found for commercial solution = 595 mg./100 ml.
(2) Mean normal for commercial solution of 630-580 = 605.
(3) Mean normal for method used in one's own laboratory of 560-630 = 595.

Hence: 606:605 : : x:595
x = *585 mg./100 ml.*

Question: A certain solution requires 23 grams of Na_2SO_4. The only form of Na_2SO_4 available to the laboratory is the hydrate Na_2SO_4: $10H_2O$. How many grams of Na_2SO_4:$10H_2O$ contain 23 grams of Na_2SO_4?

Answer:
$Na_2SO_4 : 10H_2O = 2(23 + 32 + 4(16) : 10[2(1) + 16] = 322.$
$Na_2SO_4 = 2(23) + 32 + 4(16) = 142.$
$10H_2O = 10[2(1) + 16] = 180.$
$\frac{180}{322} \times 100 = 55.9\%$ water.
$\frac{142}{322} \times 100 = 44.1\%\ Na_2SO_4.$

44.1%		559%
Na_2SO_4	•	water
23 gms.		x

44.1:23 : : 55.9:x
x = 29 gms.

Hence: 23 + 29 = *52 gms.* of $Na_2SO_4 : 10H_2O$ contains 23 gms. of Na_2SO_4.

Question: If a patient were given say 3000 ml. of 5% dextrose in 0.9% NaCl, what would be the total milliequivalent (mEq.) intake of chloride?

Answer:
(1) Every 100 ml. of the solution contains 0.9 gm. NaCl.
(2) Every 1000 ml. of a solution contains 9.0 gm.
(3) 3000 ml. contains (3 × 9.0) 27.0 gm.
(4) Since an equivalent of NaCl (M.W. 58.45) = 58.45, a milliequivalent, which is defined[1] as 0.001 equivalent, would equal 0.05845 gm.
(5) The total intake = $\frac{27.0}{0.05845} = \textit{462 mEq.}$

Question: A 1200 ml. urine specimen, sent to the laboratory, was found to contain 34 mEq./L. chloride. What would be this patient's total urinary output of chloride expressed in milliequivalents?

Answer: 34:1000 : : x:1200

$x = 40.8 = 41\ mEq.$

Or simply: Since 1200 is 1.2 times as great as one liter and 1000 ml. contains 34 mEq. chloride, then 1200 ml. contains $34 \times 1.2 = 40.8 = 41\ mEq.$

Discussion:

It is important to ask ourselves the question *Why* concerning every problem we don't understand thoroughly. It is not enough to merely follow the instructions in a procedure (adding three drops of this and three drops of that), but it is good to understand the reasons for so doing.

It is also important to prove the answers regarding problems to our own satisfaction (not merely by substituting values in a given formula and solving for the unknown or by using a correction factor); these methods give only a *solution* to the problem, not an answer satisfying to one's own mind.

Summary:

Ten questions and answers were presented: four on standard solutions, two on milliequivalents, and one each on protein-free filtrates, dye injection per unit of body weight, standardized solutions run by different methods, and hydrates.

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CONSTRUCTION OF A SLIDE RULE FOR SOLVING THE HENDERSON-HASSELBALCH EQUATION[1*]

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Frequently acidosis or alkalosis are lethal complications in a variety of clinical states. Therefore, the early recognition of an acid-base imbalance may be a vital factor contributing to a better prognosis. Accurate diagnosis of the type and degree of an acidosis or alkalosis requires solution of the Henderson-Hasselbalch equation.[3] In this relationship where

$$\text{pH} = \text{pK} + \text{Log}\,\frac{\text{serum HCO}_3}{\text{dissolved CO}_2} \qquad (1)$$

the pK is constant (6.1) while the pH, HCO_3 and the dissolved CO_2 are variables. Solution of the equation requires a knowledge of at least two of these three variables. Serum HCO_3 and blood pH determinations are easily carried out in the laboratory. However, circulating levels of the dissolved CO_2 are more difficult to estimate. Therefore, values for the soluble CO_2 fraction in the blood are usually calculated. This computation requires use of one logarithm and one antilogarithm. To circumvent these mathematics nomograms have been devised whereby amounts of dissolved CO_2 can be ascertained at any particular blood pH and serum HCO_3 concentration. McLean[5] utilizes six scales to illustrate the Henderson-Hasselbalch variables in blood while Singer and Hastings[8] propose a seven scale nomogram for acid-base assessments. It is the purpose of this report to describe construction of a simplified three scale slide rule which expresses all possible relationships of serum HCO_3, blood pH, and dissolved CO_2. This device is shown to provide an extremely rapid method for analytically evaluating acid-base status. Furthermore, a method is presented which employs the slide rule for calculating alkali deficits in cases of severe metabolic acidosis.

METHODS

Construction. The slide rule (figure 1) consists of one linear and two complete logarithmic scales of equal length. The fixed pH scale at the top of the rule is divided into linear increments of 0.01 pH units ranging from 6.10 to 8.10. Serum HCO_3 concentrations are expressed in mEq/L. on the stationary logarithmic scale at the bottom of the rule. The sliding logarithmic scale shows the dissolved CO_2 values in mEq/L. at particular serum HCO_3 levels.

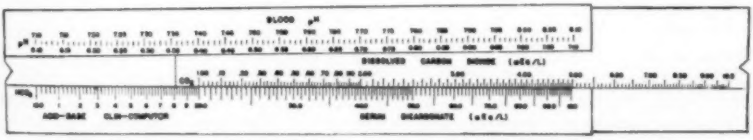

FIGURE 1. A slide rule for calculation of the Henderson-Hasselbalch variables.

[1] This work was supported in part by a research grant from the United States Public Health Service, Project A-3213, and from the Attending Staff Association of the Los Angeles County General Hospital (Unit II).
* Received for publication March 1960.

Operation. The slide rule is used for calculation of dissolved CO_2 by placing the 1.00 line of the sliding scale coincident with the predetermined blood pH value. The dissolved CO_2 concentration is then read on the sliding scale directly above the line representing the serum HCO_3 concentration as observed in the laboratory. As illustrated, the 1.00 line on the CO_2 scale has been placed directly below the line equivalent to a pH of 7.40 or 6.40. If the serum HCO_3 is 20 mEq/L. at this slide rule setting, the amount of dissolved CO_2 would be 1.00 mEq/L. When the serum HCO_3 concentrations lie within the normal range of 24 to 32 mEq/L. the dissolved CO_2 values are seen to vary from nearly 1.2 to 1.6 mEq/L. At a pH of 7.40 dissolved CO_2 values may be read directly when serum HCO_3 concentrations are 20 mEq/L. or above. To obtain a figure for the dissolved CO_2 at HCO_3 levels less than 20 mEq/L. the CO_2 sliding scale is moved so that the 10.0 line corresponds to a pH of 7.40. The decimal points on the dissolved CO_2 scale must then be moved mentally one place to the left. In a similar manner, when using pH values of 6.10 to 7.10 it is necessary to move the decimal point on the dissolved CO_2 scale one place to the right. For example, at a pH of 6.40 when the serum HCO_3 is 20 mEq/L. the dissolved CO_2 becomes 10.0 rather than 1.00 mEq/L.

DISCUSSION

By use of the proposed slide rule the soluble CO_2 fraction can be accurately calculated for all physiological combinations of blood pH and serum HCO_3. Knowledge of the concentration of this form of CO_2 becomes a useful diagnostic tool. It can be seen from the slide rule that the dissolved CO_2 content normally varies from about 1.1 to 1.8 mEq/L. when the pH lies between 7.35 and 7.45 and the serum HCO_3 values are normal. Increases in this soluble fraction of CO_2 in blood occur in respiratory acidosis while decreases are commonly found in respiratory alkalosis. Mixed respiratory and metabolic states often prove difficult to distinguish without establishing the levels of dissolved CO_2. For example, low HCO_3 concentrations are observed in pure metabolic acidosis as well as when a respiratory acidosis accompanies this condition. However, the dissolved CO_2 values do not exceed normal limits in the pure state while in the mixed acidosis this fraction may increase two to three fold. Likewise, the dissolved CO_2 may be used in a similar manner to differentiate a simple metabolic alkalosis from that associated with respiratory alkalosis. In metabolic alkalosis dissolved CO_2 levels usually remain within the normal range while they fall when respiratory alkalosis occurs with a metabolic alkalosis.

In order to maintain the blood pH within normal limits, the ratio of serum HCO_3 to dissolved CO_2 must approximate 20. In severe metabolic acidosis this ratio often falls far below this value primarily due to losses of HCO_3 and fluctuations in the amounts of CO_2 in solution. In this condition these two variables must both be considered before the alkali deficit can be properly defined. For example, the amount of $NaHCO_3$ required to bring the blood pH from 7.10 to 7.40 when the serum HCO_3 is 15 mEq/L. and the dissolved CO_2 is 1.5 mEq/L. can be calculated as follows:

$$20 = \frac{\text{serum } HCO_3}{\text{dissolved } CO_2}$$

$$20 = \frac{\text{actual } HCO_3 + HCO_3 \text{ deficit}}{\text{dissolved } CO_2}$$

$$20 = \frac{15 + HCO_3 \text{ deficit}}{1.5} \qquad (2)$$

$$HCO_3 \text{ deficit} = 20\ (1.5) - 15$$

$$HCO_3 \text{ deficit} = 15 \text{ mEq/L.}$$

This equation shows that the alkali deficit varies directly with the dissolved CO_2 as well as the circulating level of HCO_3. Haunz, Cornatzer[2] and others[1, 4] determine the need for HCO_3 by simply subtracting the serum HCO_3 concentration from the arbitrary normal value of 25 mEq/L. If this method of calculation were applied in the case cited above the $NaHCO_3$ requirement would be 10 rather than 15 mEq/L. Theoretically, the administration of the former amount of alkali is insufficient to bring the HCO_3/CO_2 ratio to 20. Therefore, the patient would remain in an acidotic state with a pH near 7.30. An equation has also been formulated by Natelson and Barbour[7] which relates blood pH directly to the HCO_3 deficiency existent in metabolic acidosis. These workers suggest that the amount of $NaHCO_3$ required to bring the hydrogen ion concentration to normal is independent of the HCO_3 and dissolved CO_2 concentrations and is a function of the blood pH only. In the patient considered above, using the Natelson-Barbour nomogram, 23.5 mEq/L. of $NaHCO_3$ would be required to bring the blood pH to normal. However, the use of this much base would result in HCO_3/CO_2 ratio of almost 26 and a pH above 7.50 according to the Henderson-Hasselbalch relationships.

The alkali deficit once established in metabolic acidosis by use of equation 2 is expressed in terms of mEq. of alkali per liter of extracellular fluid. These values can be converted into grams of $NaHCO_3$ or ml. of M/6 Na lactate by multiplying by .084 or 6, respectively. The extracellular fluid volume in liters can be estimated by taking 20 per cent of the body weight[6] expressed in kilograms. The number of liters multiplied by the alkali deficit gives the therapeutic dosages of these substances theoretically required to bring the blood pH to 7.40. The administration of repair solutions of this type are best accompanied by serial serum HCO_3 and blood pH determinations. Such analytical technic minimizes the danger of an induced alkalosis.

SUMMARY

A slide rule has been constructed which defines all states of acid-base equilibrium in terms of the Henderson-Hasselbalch relationships. This computer provides a simple yet accurate method for calculating the soluble CO_2 fraction once the blood pH and serum HCO_3 values have been established. Dissolved CO_2 values have been shown to be a useful diagnostic tool in differentiating certain mixed states of acidosis or alkalosis. A method for determining alkali deficits associated with

metabolic acidosis based upon the dissolved CO_2 as well as serum HCO_3 levels has been proposed.

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EXFOLIATIVE CYTOLOGY STATISTICAL CODING SYSTEM*†

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Because the value of Exfoliative Cytology as an aid in the detection and diagnosis of carcinoma has been well established, a routine cytology smear has become commonplace and the potentialities of the field are being further investigated. Unencumbered compilation of the data resulting from the routine examination and the many research programs now under consideration necessitates a simplified method of recording findings and maintaining statistics. It is the purpose of this article to present a cross index coding system which would provide for this need. It is assumed that every cytology laboratory maintains a record of all cases, negative and atypical. This code is of value when dealing with abnormal cytology findings.

A whole and decimal numerical system was devised whereby pertinent information could be expressed. Arrangement of the code is by *units* and *position*. A unit consists of two figures and each unit can be differentiated from a similar unit by its position. Positions are from one through four. Position one refers to the diagnosis—malignant or benign—; position two, the tissue involved; position three, the descriptive material; and position four, the fluid examined.

In addition to numbers, four letters and/or symbols placed prior to position one, or where appropriate, were used to indicate 1) (P) a prediagnosed case; 2) (PR) a prediagnosed case with radiation effect; 3) (*) a controversy in diagnosis; and 4) (:) a repetition within the *same* position.

Malignant Diagnoses	Benign Diagnoses
01. Carcinoma	50. Normal Tissue
02. Preinvasive Carcinoma	51. No Malignancy
03. Carcinoma in Situ	52. Inflammation
04. Squamous Cell Carcinoma	53. Vaginitis
05. Adenocarcinoma	54. Erosion
06. Adenoacanthoma	55. Myomas
07. Choriocarcinoma	56. Fibroids
08. Cystadenocarcinoma	57. Condylomatous Growth
09. Papillary Serous Cystadenocarcinoma	58. Polyps
10. Pseudomucinous Cystadenocarcinoma	59. Cervicitis
11. Duct and Intraductal Cell Carcinoma	60. Hyperkeratosis
12. Embryonal Carcinoma	61. Metaplasia
13. Malignant Teratoma	62. Hyperplasia
	63. Leukoplakia
	64. Endometrosis
	65. Endometritis

* From the Pathology Service, Brooke Army Hospital, Milward W. Bayliss, Colonel, M.C., Chief. This method was originally formulated by the authors and adopted for use in the Exfoliative Cytology Section, Pathology Service, Roland S. Aronson, Col., Chief., Walter Reed General Hospital, Walter Reed Army Medical Center, Washington 12, D.C.
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Malignant Diagnoses

14. Bronchogenic Carcinoma
15. Oat Cell Carcinoma
16. Small Cell Carcinoma
17. Anaplastic
18. Tumors of the Brain
19. Retinoblastoma
20. Transitional Cell Carcinoma
21. Papillary Clear Cell Carcinoma
22. Sarcoma
23. Melanoma
24. Hepatoma
25. Disseminated Lymphoma
26. Hodgkins Disease
27. Leukemia

Benign Diagnoses

66. Salpingitis
67. Papilloma
68. Fibroadenoma
69. Adenomyosis
70. Pregnancy
71. Decidual Reaction
72. Benign Teratoma
73. Bronchitis
74. Pneumonitis
75. Pleuritis
76. Gastritis
77. Ulcer

Tissue Involved

.01 Vulva
.02 Labia
.03 Vaginal
.04 Uterus
.05 Cervical
.06 Endocervical
.07 Endometrial
.08 Fundus
.09 Myometrium
.10 Cervical Stump
.11 Ovary
.12 Breast
.13 Penis
.14 Testis
.15 Prostate
.16 Lung
.17 Right Lung
.18 Left Lung
.19 Bronchus
.20 Right Bronchus
.21 Left Bronchus
.22 Pleura
.23 Scalene Node
.24 Brain
.25 Ear
.26 Neck
.27 Optic Nerve
.28 Sinus
.29 Lingula
.30 Tongue
.31 Larynx
.32 Esophagus
.33 Stomach
.34 Peritoneal
.35 Omentum
.36 Recto-Sigmoid
.37 Left Kidney
.38 Right Kidney
.39 Bladder
.40 Liver
.41 Pancreas
.42 Lymph Nodes
.43 Bones

Descriptions

.00 01 Preinvasive
.00 02 With Glandular Involvement
.00 03 With Stromal Invasion
.00 04 Invasive
.00 05 Well Differentiated
.00 06 Differentiated
.00 07 Poorly Differentiated
.00 08 Undifferentiated
.00 09 Grade 1
.00 10 Grade 2
.00 11 Grade 3
.00 12 Grade 4
.00 13 With Extension
.00 14 Infiltrating
.00 15 Far Advanced
.00 16 Generalized
.00 17 With Radiation
.00 18 Metastatic
.00 19 Primary Site Undetermined
.00 20 Primary Site Preceding
.00 21 Unoperable
.00 22 Residual
.00 23 Recurrent
.00 24 Mild, slight, poor, minimal
.00 25 Moderate
.00 26 Chronic
.00 27 Acute
.00 28 Irregular
.00 29 Marked, severe, intense
.00 30 Atypical
.00 31 Focal
.00 32 Superficial
.00 33 Hyperplastic
.00 34 Hamartoma

Fluids

.00 00 01 Sputum	.00 00 10 Breast Fluid
.00 00 02 Bronchial Washings	.00 00 11 Gastric Lavage
.00 00 03 Ventricular Fluid	.00 00 12 Ascitic Fluid
.00 00 04 Spinal Fluid	.00 00 13 Gall Bladder Fluid
.00 00 05 Sinus Fluid	.00 00 14 Urine
.00 00 06 Tracheal Fluid	.00 00 15 Ovarian Fluid
.00 00 07 Esophageal Fluid	.00 00 16 Lymph Node Aspiration
.00 00 08 Pleural Fluid	.00 00 17 Origin not known, Miscellaneous, or direct smear
.00 00 09 Pericardial Fluid	

A 3 x 5 Master Index Card used to denote a malignant or benign diagnosis should contain the name, corresponding code, accession number, surgical and/or autopsy number of every patient having the designated diagnosis. This master index card is filed numerically in accordance with the diagnosis.

SQUAMOUS CELL CARCINOMA 04. Card No. 1

Smith, Mary	04.05 04	S 00000	C 00000	C 00000
Doe, Jane	04.16 05	S 00000	C 000000	C 00000
Jones, John	04.31	S 00000	C 000000	C 00000

Fig. 5—Master Index Card

The individual Patient Card which identifies the patient contains the accession number of the smear and its finding, the surgical and/or autopsy number, the complete code of all diagnoses concerned and the dates of these procedures. These cards are filed alphabetically.

Susp Cx 05.07 18

JONES, NANCY

C 000000	10-20-58	Susp Cx
S 000000	10-26-58	05.07 18

Fig. 6—Individual Patient Card

Color separator cards in a small container were used for malignant (yellow) and benign (blue) diagnoses in the Master Index File.

The Individual Patient Cards require the utilization of four sets of alphabetical separators. A *pending* set contains cards of patients with abnormal smears which have not as yet been diagnosed. These become *active* when a diagnosis has been established by surgery. It is wise to maintain a dead file for those patients on which no surgery has been performed at the end of six months. The diagnosis may be made or confirmed at death by autopsy and at this time the patient's card is placed

in the *deceased* file. Posting of these files can be done conveniently on a weekly, bi-weekly, or monthly basis.

A complete series of numbers would appear:

Diagnosis
Tissue
Description
Fluid

00.00 00 00
1 2 3 4 Positions

After position two (2) a space is left between each succeeding position.

Examples:

1) Well differentiated invasive squamous cell carcinoma of the cervix; 04.05 04:05
   The colon shows a repetition of position 3 (description)
2) Carcinoma in Situ with glandular involvement: 03.00 02
   Tissue site was not indicated, therefore a double 0 is placed in position 2 (tissue)
3) Metastatic Adenocarcinoma of the lung with the liver as the primary site. Abnormal cytology of Sputa and Bronchial washings was reported. 05.16:40 20:18 01:02
   Position 2 is repeated as shown with a colon, and also position 3. The first unit of position 3 identified the primary site of the cancer to be the last unit of the tissue (2) position. Position 4 (fluid) repeats and lists the two fluids interpreted as abnormal.
4) Prediagnosed Adenocarcinoma of the Endometium: (P) 05.07
5) Severe chronic cervicitis: 59.00 26:29
   A repetition of position 3 denotes two descriptive terms.

In some cases the patient may have more than one diagnosis and all of these would appear on the Patient's Individual Card. The Master Index would only show the diagnosis corresponding to that card.

The diagnosis unit is divided into 2 parts; 01. through 49. and 50. through 99. As with the other positions, none has its full quota, therefore each unit can have an additional diagnosis, tissue, description, or fluid as the situation demands.

Since this is a numerical system, it is felt that it can easily be adapted to automation. For some laboratories the first two positions may be sufficient.

This system has been used successfully in coding 1500 tissue diagnoses relating to Exfoliative Cytology examinations and in the preparation of papers for publication.

Summary

A numerical Cross Index Coding System for maintaining Exfoliative Cytology records and statistics is presented. The system is arranged in units and positions which differentiate specific terms. Several example cards and diagnoses are offered for better understanding. It is felt that this method is flexible and can be adapted to automation. 1500 tissue diagnoses have been successfully coded and the preparation of scientific papers expedited.

TRANSFUSION IN MULTIPLE-ANTIBODY IMMUNIZATION*

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The incidence of sensitization of the Kell factor occurring simultaneously at the height of a sensitization to the Duffy, Fy^a, factor is reportedly rare.[2,12,14,22] More rare is the recognition of this combination of sensitizations in the clinical work of a small hospital laboratory. That the challenge of a problem of multiple antibodies may be met equally as well in the 65-bed hospital with its necessarily limited scope of activity, as in the specialized facilities of the large hospital, derives from the cooperative effort of the ancillary medical profession to adopt the most recent scientific advances to ordinary patient care. In the identification and greater availability of the rare test cell antigens and antibodies, medical research has given the clinical laboratory (1) tools with which to determine causative factors in transfusion incompatibility and hemolytic disease of the newborn, and (2) effective instruments to prevent these reactions. Their use demands the professional skill of technologists, who are cognizant of the newest developments in transfusion therapy, whose laboratories are equipped to perform the essential blood grouping techniques, and who have available for consultation the highly specialized resources of a dependably administered reference blood center. The purpose of this paper is to show how these factors were coordinated in the apparently ordinary request to transfuse a surgical patient.

Medical History

The history of transfusion reactions, jaundice, and hemolytic disease of the newborn were negative factors in the medical record of this individual. The patient, a white female, age 29 years, of average good health, was admitted to the hospital for exploratory surgery of the common duct. On the second post-operative night, she developed symptoms of profound shock. The hemoglobin dropped from the admission level of 12.8 grams/100 ml. to 11.7 grams/100 ml., with a corresponding decrease in the hematocrit and red cell count. One transfusion of 500 ml. of whole blood was given. The unit was received without incident, with no antibody response apparent. Hemoglobin and hematocrit values 48 hours following revealed not the expected increase, but a significantly lowered hemoglobin value of 9.4 grams/100 ml. with a hematocrit of 34 vol.%. Evidence of hemorrhage could not be found to account for the decreased value.

A second transfusion of 500 ml. of whole blood was given three days later. No incompatibility was noted in the crossmatching procedure and the patient experienced no difficulty in receiving the unit of blood, until the last 100 ml. was reached. At this time she had a severe chill, accompanied by dyspnea and a rise in oral temperature to 102°F. The blood was discontinued immediately and returned to the laboratory for recheck typing and crossmatching. At this time the hemoglobin and hematocrit values were approximately those obtained after the administration of the first unit of blood, 9.7 grams/100 ml. and 35 vol.% respectively.

* Received for publication June 1959.

The ABO and Rh groupings, both of the donor and recipient bloods, were carefully reexamined to determine whether mistyping or incorrectly identified units had been responsible for the reaction. The compatibility procedure, employing the saline, albumin and indirect Coombs tube techniques,[23] revealed again a correctly matched unit insofar as the in vitro testing could confirm. The nature of the patient's illness seemed the factor largely responsible for the reaction.

The symptoms of this reaction were exhibited for a brief time, and the usual tests on pre- and post-transfusion blood to determine the presence of hemoglobinemia and/or increased bilirubinemia revealed no elevated values.[7,16]

A few days later, use of a new specimen of the patient's serum in crossmatching a third unit for transfusion produced marked agglutination in the major saline and indirect Coombs sensitivities. When compatibility of a second, type-specific unit was proved in the three sensitivity procedures, this agglutination was not thought to be of major significance, the previous transfusion reaction being attributed largely to interfering substances in the patient's serum, rather than to the possibility of sensitization.[5] Administration of this compatible unit of blood produced no apparent hemolytic or pyrogenic response, with pulse, temperature, and blood pressure readings remaining normal throughout the transfusion.

Following a fourteen-day hospitalization, the patient was dismissed to her home for ten days. She was readmitted to the hospital, critically ill.

With an admission hemoglobin of 11.7 grams/100 ml., clinical and radiological studies indicated the necessity of emergency surgery, for which the laboratory was requested to have available two compatible units of blood.

Because the patient had been previously phenotyped a group A, DCE/c, no difficulty in preparing the two units of blood was anticipated. Results of the crossmatching, however, using type-specific units, revealed agglutination of varying degrees in the saline, albumin and Coombs major crossmatchings.

Associating the agglutination with the extremely toxic condition of the individual, the technologist repeated the crossmatching sensitivity, using two group A dce units. After the required incubation period, agglutination was again observed in all major crossmatchings. Four group O DCE units were next examined for compatibility. The same marked agglutination, previously observed with the type-specific cells, resulted. With group O dce blood the last possibility, crossmatching with four of these units revealed the same pattern of clumping in each of the major crossmatching media, as noted above. Demonstration of this identical agglutination in matching these units exhausted the available supply of blood in the hospital bank.

The repetition of the pattern of agglutination in each major crossmatching with cells of the various Rhesus phenotypes indicated the presence of a potent antibody whose nature required further investigation. Notification of the immediate inability to furnish compatible blood for the patient was made to the attending surgeon, and efforts to obtain blood from other sources were initiated.

Methods

The surgical procedure revealed an acute, hemorrhagic, necrotic pancreatitis to be the cause of the undetermined hemoglobin and red cell loss. With a further hemoglobin decrease to 7.4 grams/100 ml., the assistance of a reference blood center nearby, was sought.

Within 18 hours the reference blood center reported the following causes of the crossmatching difficulty:

1) sensitization to Duffy, Fy^a, factor
2) presence of a non-specific cold agglutinin
3) presence of an indeterminate sub-group of a A antibody

With the detection of the non-specific cold agglutinin in addition to the Duffy sensitization, it became necessary to determine compatibility by performing each crossmatching at 37°C.[4,15,17] The A sub-group sensitization further necessitated a careful washing of the test cells in order to rule out pseudoagglutination.[2,10]

Shortly after identifying the factors responsible for the crossmatching problem, the reference blood center found the first type-specific unit showing no agglutination with the recipient's serum. Verification of the compatibility could not be made in the hospital laboratory until eight hours later, when the effects of Dextran had been lessened. Compatibility was successfully established, using a triple-washed saline cell suspension while performing the entire procedure at 37°C. This first unit of Duffy-negative blood was received by the patient with no evidence of transfusion reaction—exactly 45 hours after the first signs of incompatibility had been recognized.

From a choice of 10 Duffy-compatible units obtained in the examination of 146 bloods, the patient received 3 type-specific units on the second, sixth, and eleventh post-operative days, respectively.

The degree of agglutination observed in the crossmatching techiques with the various donor bloods ranged from microscopic to grossly visible clumping. In most instances, where moderate agglutination occurred in the saline sensitivity, a small degree of agglutination was noted in the albumin crossmatch, but in every case, the agglutination was most marked in the indirect Coombs major set-up. The fact that these reactions could not be attributed to the same agglutinin, introduced the necessity of screening so large a number of individuals in order to find suitable donors who did not possess the antigens corresponding to these particular immune antibodies.

In a routine anti-Fy^a titration performed approximately three months following the patient's reception of the Duffy-negative blood, evidence of a new antibody was noted in a cell panel study of the Duffy-sensitized serum. This new antibody proved to be a potent agglutinin, exhibiting a significant titer with type-specific cells. Further investigation showed that the new substance was readily detectable in the saline medium, as well as in the indirect Coombs procedure.[8] Antibody screening tests employing erythrocytes of various antigenic combinations demonstrated this new antibody to be a potent anti-Kell agglutinin. The reference blood center, in determining the nature of the original Duffy antibodies, selected cell types which ruled out the presence of the Kell agglutinin in the initial antibody study. In the urgency, however, to find compatible

Duffy blood, the Kell factor was not determined on the Duffy-compatible donors, because of a temporary shortage of Kell typing serum. Thus, in the effort to satisfy the demand for Duffy-negative blood, Kell-positive blood was administered.

The strength of the Kell agglutinin was determined by titration with the cell factors which reacted positively in the antibody screening procedure. The titration scores are listed in Table 1.

TABLE 1—Recipient Titration Scores With Kell-Duffy Cell Panel*

Cell	Saline	Indirect Coombs	Albumin	Indirect Coombs
K+, k—, Fy (a—)	1=4	1=16	1=1	1=16
K—, k+, Fy (a+)	0	1=8	0	1=64
K+, k+, Fy (a+)	1=4	1=16	1=1	1=16

* Data of the reference blood center.

Neither evidence of the antibody development nor symptoms of transfusion reaction or hemolytic response were exhibited by the patient, despite the other sensitizations she already possessed, and the potency of the Kell factor in this respect.[1,9,11,14,19,22]

Discussion

Analysis of the clinical history, the compatibility studies, and the above data point up the following possible routes of sensitization in this antibody complex.

Duffy Factor

Cell panels employing specific Duffy, Fy(a+), cells demonstrated the presence of this immune antibody in the patient's serum, while the use of anti-Fy^a serum definitely established the presence of this factor on the erythrocytes of her husband and of her first child. Since the patient had received no transfusions previously, it seems obvious that her original immunization to this factor occurred in the first pregnancy, nine years ago. No evidence of hemolytic disease of the newborn was seen in this infant.[11] The Duffy typing of her second child was found to be negative, indicating that reimmunization to this factor did not occur.

Following post-partum hemorrhage, three transfusions, designated simply group A, Rh positive in 1951, were received by the patient with no apparent difficulty.

Within the following seven-year period the patient had undergone minor surgery (1954), at which time she received no transfusions.

It is postulated that the second of the three units of blood received in the first of her current admissions was undoubtedly a Duffy positive, Fy(a+); because it was impossible to locate the donor for confirmation tests, the presence of the Duffy antigen in this unit was not definitely established by experimental study.

It is of interest to note that the patient, as well as her mother and sister were found to be Duffy negative, Fy(a—). Her father, now deceased, must have been a Duffy positive, Fy(a+), since her three brothers all were shown to be Duffy positive, Fy(a+). Parenthetically, the

Duffy negative sister is also married to a Duffy positive, Fy(a+), individual and none of their three children has shown evidence of hemolytic disease.[12, 18, 21, 22]

The finding of a moderately high-titred antibody in an individual, having received a total of three transfusions in the immediate postpartum period nine years previously, and two in the current hospitalization, agrees with the calculated percentage of probability.[7] Although the Fy^a allele may be found in 65% of the Caucasian race, sensitivity to this factor (though of much more common incidence than to its counterpart, Fy(b+)), is still regarded of comparatively rare occurrence.[2, 12, 13, 14, 18]

Since the discovery of the Duffy, Fy^a, antigen by Cutbush, Mollison and Parkin in 1950, and its reciprocal factor, Fy^b, by Ikin in 1951, a total of ten to fifteen cases of sensitization to Fy^a has been reported in the literature.[12, 13, 16, 21] Almost all cases of Duffy sensitizations reported to date, have occurred in response to antigens of transfusion origin; those caused by fetal immunization have been much fewer in number.[14]

Because the antigenicity of the Duffy, Fy^a, factor is less than that of the stronger Rhesus antigens, immunization to the factor as a single sensitization is less often observed than in combination with other sensitizations.[20, 21] Results of Kell sensitization, in the work of certain investigators, occurring in the presence of anti-Fy^a sensitization, resulted from the transfusion of Duffy-compatible blood in which the Kell factor had not been ruled out.[12] As in the instance recorded above, the administration of type-specific Fy(a+) compatible blood, which was Kell positive, initiated this antibody formation.

Sensitization to the Kell factor has been rated by prominent authorities working with varied sectors of the population in the United States first and second in a group comprising the so-called "rare" antibodies, i.e., Kell, c, E, Fy^a, Le^a, P, Jk^a, k, and C.[24]

Summary

1. The problem of Duffy sensitization occurring as part of an antibody-complex in the serum of a critically-ill patient requiring immediate transfusion is presented.
2. Methods of securing Duffy-compatible blood are given. From direct matching screening of 146 bloods, 10 Duffy-compatible donors were found.
3. Follow-up study of the Duffy sensitization revealed the presence of a new and potent agglutinin, anti-K, occurring simultaneously with anti-Fy^a.
4. The available data is analyzed briefly.

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FOUR NEW LAB PROCEDURES FOR THE MODERN LABORATORY*

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The tremendous number of new laboratory procedures that are published annually continue to appear in all our journals at an ever increasing rate. It is the problem of the pathologist and the technologist to try to evaluate these tests and to incorporate them in their own laboratory whenever practical. In considering new laboratory tests, there are several criteria that should be utilized.

1. Does the new test solve some diagnostic problem that is not already being taken care of by a test that is in current use.
2. Can the test be performed easier or more rapidly.
3. Is the test more accurate than some test that is now being done.
4. Is the test less expensive to perform than present day tests.

In general if the newer test meets any of these criteria, it is probably worth while to institute it in your laboratory. If it meets two or more of the above criteria, then it is probably very urgent to include it in your laboratory. The only exception would be in some tests which actually are only rarely used and which would be better left in larger reference laboratories where the tests would be performed more often. It is probably unwise for smaller laboratories to set up tests that will not be done fairly frequently as the technologists will not become sufficiently well acquainted with the procedure to do it well. The cost of chemicals will occasionally be prohibitive when a test is rarely used.

Listed below are four laboratory procedures that are relatively new and which fulfill one or more of the above criteria. The leucine aminopeptidase test is apparently a fairly accurate method of ruling out carcinoma of the head of the pancreas. The uropepsin test is helpful in evaluating the hydrochloride secretion of the stomach in patients who are too ill to have a tube attached or even too ill to have an oral diagnex test. Although the occasions when this test will be used are relatively infrequent, occasionally the test will be the only method to differentiate the cause of massive gastrointestinal bleeding. The serotonin test and the urine catecholomines are tests used to diagnose specific type tumors. These tumors are relatively rare, but once again, the use of these tests is the only accurate method of making the diagnosis prior to surgery.

Leucine Aminopeptidase

Leucine aminopeptidase is a proteolytic enzyme that is capable of hydrolyzing L-leucyl peptide. It is widely distributed in plants and animals and is found regularly in the gastric juice, bile and duodenal secretions, in pleural, peritoneal and spinal fluid and in the urine and serum of normal people. Its intracellular function appears to be in the hydrolysis of peptides, and transferring L-leucine from one peptide molecule to another. The enzyme can be measured both in the serum and in the urine and it is expressed in units. A unit of leucine aminopeptidase activity is defined as the number of milligrams of beta-napthyl-

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amine liberated in a 24 hour volume of urine or as micrograms liberated by serum or tissue activity. The normal range in the urine is as follows:

Men—50 to 125 units

Women—20 to 70 units

Normal range in the serum is:

Men—75 to 200 units

Women—80 to 200 units

Leucine aminopeptidase activity is increased during pregnancy with a progressive fall in activity in the immediate post partum period. The activity is increased both in urine and in the serum in post-operative patients. This increase is non-specific and occurs both in patients with tumors and benign conditions. In non-cancer patients this increase is of short duration and returns to normal in 15 to 30 days. In cancer patients the enzyme will often remain elevated and will often be elevated in the urine prior to operation but is usually normal in the serum. (Serum may have normal activity when the urine activity is high; however, when serum activity is increased, the urinary activity is always increased.) In patients with cancer, other than carcinoma of the pancreas, the leucine aminopeptidase activity is almost invariably normal in the serum and shows elevation in the urine in only about 50% of cases. These elevations in the urine are only moderate. However, if there is metastasis to the liver from any tumor, then there will be elevations both in the urine and the serum and these can reach high levels of 400-600 units.

Patients with pancreatitis and with common duct stone with partial obstruction also showed moderate elevations in serum activity. However, these elevations were of relatively low values and they were not persistent and returned to normal within a period of a few days. Cholecystitis seldom caused any increase in activity. Patients with lymphoma and leukemia had slightly increased urinary activity but normal or slightly elevated serum levels.

The most significant changes in the activity, both in the urine and in the serum, were found in patients with carcinoma of the pancreas, particularly of the head of the pancreas. In all such patients this aminopeptidase activity showed striking elevations. These elevations were above 400 units in the serum and with moderately elevated values in the urine. This elevation is also seen in patients who have carcinoma of other organs which is secondarily invading the pancreas. Patients with carcinoma of the tail of the pancreas show moderate elevation but they are not as striking as those with carcinoma of the head of the pancreas. These elevations in carcinoma of the pancreas were seen in patients with early disease who did not have symptoms referable to their disease, without jaundice and with normal serum alkaline phosphatase levels. This assay is especially valuable in ruling out carcinoma at the head of the pancreas as there were no instances of carcinoma of the head of the pancreas that did not show the marked elevation.

Briefly, in summary then, it can be said that the finding of a high serum leucine aminopeptidase activity is strong laboratory evidence for a carcinoma at the head of the pancreas or for carcinoma from other primary sites that has metastasized to the liver. The finding of normal

values fairly well rules out this diagnosis. Moderate elevations are occasionally seen in the urine in case of cancer in other sites but in these patients the serum level is normal. Transient serum elevations are seen in acute pancreatitis and rarely in cholecystitis. Leukemia and lymphoma give elevated urinary activity but normal or only slightly elevated serum levels (*Cancer*, March, 1958, page 283).

Uropepsin Test

The level of uropepsin excretion in the urine has been shown to correlate closely with the level of gastric acid secretion and gastric peptic activity. It has the advantage that the patient can follow his usual routine, with no fasting and no medication and no intubation being necessary.

The patient is instructed to collect a urine specimen for a definite period of time (4 hours is preferred and the timing should be *exact*). Collect in a bottle with 0.5 cc. of 25% sulfuric acid. If the specimen is to be shipped, toluene should be added as a preservative.

This test has proven valuable in the following differential diagnosis.

1. Gastric peptic ulcer versus gastric carcinoma (high levels in ulcer and low or absent in carcinoma).

2. Macrocytic anemias (absent in pernicious anemia and no increase when stimulated by histamine).

3. Massive hematemesis (low levels consistently found in bleeding esophageal varices and high in bleeding duodenal ulcer).

The normal range is about 40-150 micrograms per hour with the mean being about 80 micrograms per hour.

Serotonin

Serotonin is produced by the argentaffin cells of the gastrointestinal tract. These cells are also the ones that give rise to carcinoid tumors. Most carcinoids originate in the region of the ileocecal valve but can be anywhere in the G.I. tract. Carcinoid tumors only give generalized symptoms of flushing (acute reddish flushing of the face with occasional spread over the chest, arms and legs) when they have metastasis to the liver.

Serotonin (5-hydroxytryptamine) is derived from the amino acid tryptophane. (Pellagra is sometimes seen in carcinoid syndrome because of diversion of tryptophane to serotonin rather than to nicotinic acid as in the normal patient. Normally only about 1% of tryptophane is converted to serotonin but in carcinoid syndrome, approximately 60% is converted to serotonin.) The pharmacologic effects of serotonin are variable depending on the species and the state of the individual. The blood pressure is elevated in some and lowered in others. Pathology: There is a thickening of the endocardium especially on the right side of the heart and this results in deformities of the pulmonary and tricuspid valves with stenosis and insufficiency. This thickening is apparently due to the direct action of serotonin on the endocardium, and is partially prevented in the left side of the heart by the destruction of the serotonin by amine oxidase action in the lungs.

Values of serotonin in the serum:
Normal—0.1 to 0.3 microgram per cc.
Metastatic carcinoids—0.5 to 217 micrograms per cc.
5-HIAA in urine (5-hydroxyindole-acetic acid.
Normal—2 to 7 milligrams per day.
Metastatic carcinoids—0.5 to 217 micrograms per cc.

There is a simple screening test for elevated values of 5-HIAA in the urine but a more difficult and more definitive test is needed for exact values. Despite the fact that these values are usually not so markedly elevated and the usual syndrome is not seen except in cases where there are metastases, the diagnosis is necessary and diagnostic procedure should be carried out because these patients should be treated even in the presence of metastasis. Radical surgical removal is indicated because it will give a decrease in the symptomatology and the tumor is very slow growing and the patient will often live ten to twenty years even following the diagnosis. In addition to the surgical removal these patients receive some benefit from chlorpromazine. The presence of carcinoid tumor should be suspected in the tests run in the following conditions:

1. In the presence of the full flushing syndrome.
2. Intermittent, isolated episodes of flushing.
3. Intestinal obstruction in the ileocecal region.
4. Hepatomegaly of unexplained cause.
5. Valvular heart disease of unexplained cause, especially involving the pulmonary and tricuspid valves.

Norepinephrine and Epinephrine
(pressor amines)

Pressor amines are produced in excessive amounts in some pheochromocytomas, most of which occur in the adrenal gland. Pheochromocytomas are derived from the chromaffin cells which, microscopically, look somewhat like large ganglion cells. When these tumors are functioning, they have a sympathomimetic action. If functioning they may cause intermittent attacks or persistent attacks of hypertension. In addition to the adrenal glands, the tumors are also found in the retroperitoneal space and in the mediastinum and they are occasionally bilateral in the adrenal glands.

Two large groups of tests are run: (1) Drug test. (2) Pressor amine determinations.

Drugs—In testing for pheochromocytoma by drugs, an attempt is made to lower the pressure if it is elevated or to elevate the pressure if it is lowered. All other drugs should be stopped for at least two days prior to the test. (Apresoline should be stopped for a period of 14 days prior to the test.) The drug to be used depends entirely on the basal blood pressure. If the blood pressure is less than 170/110, histamine is the drug of choice. (0105 milligrams histamine base in 0.5 ml. isotonic saline given rapidly intravenously.) The test is considered positive if the blood pressure rises to a maximum in two minutes greater than the rise seen in the cold pressor test. The cold pressor test must be done and the results compared or the histamine test is of no value.

Phentolamine is the drug of choice if the blood pressure is greater

than 170/110, especially in persistent hypertension. A positive test is indicated if the blood pressure falls 35 mm systolic and 25 mm diastolic and remains depressed for 3-4 minutes. Five milligrams of phentolamine should be injected rapidly in the test. One can often get a positive test in the same patient with both of these drugs used at different times. No single test is completely reliable. If the tests above are negative or doubtful, the pressure amines should be determined.

Pressor amines—The pressor amines should be determined on blood at the height of the blood pressure elevation. If elevations are not occurring spontaneously, take the specimen at the height of the blood pressure elevation following the histamine test. (Urine values can also be determined. The urine specimen should be collected following an episode of hypertension or following injection with histamine.) The determination of the pressor amines is the most accurate method of diagnosis. Concentration of pressor amines is nearly always less than 3.5 micrograms per liter of plasma in essential hypertension. It is almost invariably elevated in pheochromocytoma. However, false positives will occur in renal insufficiency, jaundice, increased intracranial pressure and lymphoma so the entire patient must be evaluated and not simply the pressor amine results.

These test for pheochromocytoma should be done in the following type patients:

1. Patients who complain of spells of headache, associated with perspiration, thoracic and abdominal pain, nervousness and basal motor phenomena.
2. Thin patients with a history of fluctuating hypertension. All other thin people with hypertension should undergo these tests as this lesion rarely occurs in obese patients and practically all these patients are very thin for their height.
3. All young individuals with hypertension should be evaluated.
4. Individuals with hypermetabolism without hyperthryoidism should be tested.
5. Individuals with a short history of hypertension.
6. Individuals with severe hypertension who do not fall into the other groups.
7. Patients who respond unsatisfactorily to anesthetic and who get a rise in blood pressure during anesthesia. The anesthesia should always be stopped in these patients.

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A NEW METHOD FOR PERFORMANCE OF TOTAL AND DIFFERENTIAL CELL COUNTS IN CEREBROSPINAL AND SEROUS FLUID*

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Introduction

Cell counting in various body fluids, other than blood, has often presented problems because of the paucity of cells (leukocytes), the admixture of blood, and the difficulty encountered in the performance of differential counts. Diluting fluids for leukocyte counts in whole blood are usually unsatisfactory for this purpose since they require the use of a small amount of sample mixed with a relatively large amount of diluting fluid, resulting in a large dilution factor. Leukocyte counts in body fluids, as cerebrospinal fluid, are usually much lower than in whole blood, and the dilution of the specimens 1:20 or 1:10 with whole blood diluting fluid results in significant errors and loss of ability to detect slight increases in leukocyte counts.

Method

A new dye preparation, Thedane Blue,† designed for the identification of blood parasites, offered interesting possibilities. This product contains methylene blue for staining and saponin for hemolysis. Thedane Blue was found to be an excellent diluting fluid for cell counts in cerebrospinal and serous fluids. The leukocytes are well stained in the hemocytometer chamber and direct differential counting is greatly facilitated. Thedane Blue is available in two strengths, T3 and T5. T3 is generally satisfactory, but for viscid fluids, the stronger T5 is preferred.

Presently we use 1 part of dye solution to 9 parts of well-mixed fluid in a WBC pipette. The leukocytes in all nine squares of the hemocytometer chamber are counted, and the following calculations are made:

1. WBC/c.mm. = WBC in 9 sq. mm. $\times \frac{10}{9} \times \frac{10}{9}$

To another counting chamber, the well mixed fluid is added as received.

2. Total cell count/c.mm. = Total cells in 9 sq.mm. $\times \frac{10}{9}$

3. RBC/c.mm. = Total cell count/c.mm. — WBC/c.mm.

Occasionally, the leukocytes are overstained. In these instances, the count is repeated, drawing the diluting fluid to the 0.5 mark, then drawing the sample in to the 11 mark. In this instance, equation 1 above becomes:

$$\text{WBC/c.mm.} = \text{WBC in 9 sq.mm.} \times \frac{10}{9} \times \frac{20}{19}$$

For cerebrospinal or serous fluid containing a large amount of blood or many leukocytes, the specimen may be treated exactly as whole blood, using the usual whole blood diluting fluids.

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** Dr. Delvaux is Assistant Pathologist; Miss Forsyth is Section Chief, Bacteriology Division; Miss Renfroe is Chief Technologist.
† Obtainable from national Analine Division, Allied Chemical and Dye Corporation, 40 Rector Street, New York 6, N. Y.

FUNDAMENTAL MECHANISMS OF ENZYME ACTION AND THEIR IMPORTANCE TO THE MEDICAL TECHNOLOGIST*

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The problem in the enzyme field which is generally of greatest interest to the medical technologist is that of enzyme assay. The question is usually not one of calculating the number of moles of enzyme actually present in a sample, but rather expressing the enzyme activity in relation to some arbitrary standard. Regardless of how the analytical determinations are actually done, all procedures essentially measure rates; the higher the rate at which the substrate is transformed, the more enzyme is considered to be present. In order to be sure that our assay technique is valid, it is necessary for us to consider the conditions which are necessary for the rate of the enzyme catalyzed reaction to be directly proportional to the enzyme concentration.

A great deal of light was shed on the mechanism of simple enzyme reactions by Michaelis and Menten (1). This theory, or modification of it, has been repeatedly substantiated by experimental means. Michaelis and Menten suggested that enzyme and substrate molecules come together to form an enzyme-substrate complex ES, and that this process very rapidly reaches equilibrium. The enzyme-substrate complex then decomposes to form free enzyme and products.

$$E + S \overset{\overline{K}}{\rightleftarrows} ES$$

$$ES \xrightarrow{k2} E + \text{Products}$$

where $\overline{K}$ is the equilibrium constant for the first reaction and k2 is a rate constant. This rate constant k2 may be considered to be a proportionality constant relating the rate of decomposition of ES complex to the concentration of ES complex. The rate v— of the above reaction is given by v— = k2 [ES] (1) Brackets denote concentrations in all cases.

Using conventional procedure, the equilibrium constant $\overline{K}$ may be written

$$\overline{K} = \frac{[ES]}{[E]\,[S]} \qquad (2)$$

Let [E]o be equal to the concentration of free enzyme at the beginning of the reaction and [E] the concentration of free enzyme.

$$[E]o = [E] + [ES]$$

or

$$[E] = [E]o - [ES] \qquad (3)$$

substituting equation (3) in equation (2)

$$\overline{K} = \frac{[ES]}{([E]o - [ES])[S]}$$

* Reprinted with permission from The West Virginia Microscoop, Vol. 2, No. 4, Oct. 1959.

and solving for [ES] by ordinary algebra

$$[ES] = \frac{\overline{K}[E]o[S]}{1 + K[S]} \tag{4}$$

However, we said that the velocity of the enzyme catalyzed reaction was expressed by equation (1)

$$v\text{—} = k2[ES]$$

so now making appropriate substitution

$$v\text{—} = \frac{k2\,\overline{K}[E]o[S]}{1 + \overline{K}[S]} \tag{5}$$

This is the well known Michaelis-Menten rate equation, and states that the rate of an enzyme catalyzed reaction is dependent upon:

1) the rate constant k2 for the decomposition of the enzyme-substrate complex.

2) the equilibrium constant $\overline{K}$ for the reaction

$$E + S \underset{}{\overset{\overline{K}}{\rightleftarrows}} ES$$

3) the enzyme concentration

4) the substrate concentration

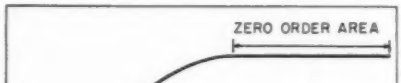
It should be noted here that Michaelis and Menten originally wrote their equilibruim constant

$$Km = \frac{[E]\,[S]}{[ES]}$$

$$\text{so our } \overline{K} = \frac{1}{Km}$$

where Km is the so called Michaelis constant.

The Michaelis-Menten equation explains a well known phenomenon. When all conditions, including enzyme concentrations, are kept constant, and the substrate concentration varied, the type of curve obtained is shown in figure 1.



It can be seen that at low substrate concentrations increasing the concentration of substrate increases the rate of the reaction. When the substrate concentration is increased to higher levels, it is noticed that the rate tends to become independent of substrate concentration until eventually adding more substrate has no further effect on the rate. The reaction is now called zero order.

Examination of equation (5) makes it possible to see how such a situation comes about.

1) At low substrate concentrations.

If the substrate concentration is very low, it follows that the term $\overline{K}[S]$ in the denominator of equation (5) becomes smaller in relation to one until finally the rate expression becomes approximately

$$v_{-} = k2\,\overline{K}[E]_o[S] \tag{6}$$

Thus equation (6) informs us that at low substrate concentration, the rate is dependent on $k2\overline{K}$ (constants for our particular enzyme and substrate combination) and on **both** the **enzyme and substrate** concentrations.

2) At high substrate concentrations.

If the substrate concentration is very high, then the $\overline{K}[S]$ term in the denominator of equation (5) becomes very large in relation to one. At very high substrate concentrations, the term one in the denominator of equation (5) may be, to an approximation, ignored and the rate equation becomes

$$v_{-} = \frac{k2\,\overline{K}[E]_o[S]}{\overline{K}[S]}$$

and upon simplifying this fraction we obtain

$$v_{-} = k2[E]_o \tag{7}$$

It is seen from equation (7) that under these conditions at high substrate concentration the rate is proportional to

1) k2, the rate constant for the decomposition of ES complex. This will remain the same as long as we deal with the same enzyme and substrate.

2) $[E]_o$ the **enzyme concentration**

Hence we see that **high substrate concentration** is the condition which is **necessary** for the rate of an enzyme-catalyzed rection to be **directly proportional to the enzyme concentration.**

The physical significance of equation (7) is quite easy to see. If we increase the substrate concentration to very high levels, the equilibrium

$$E + S \rightleftarrows ES$$

is forced toward the right until all of the available enzyme is tied up as enzyme-substrate complex. Therefore the only step which controls the rate of the reaction is

$$ES \xrightarrow{k2} E + \text{Products}$$

Since the concentration of ES is now equal to Eo, the concentration of enzyme present in the beginning, the rate is equal to

$$v_{max} = k2[ES] = k2[E]o$$

and we say that the enzyme is **saturated** with substrate.

Michaelis and Menten originally considered

$$E + S \underset{}{\overset{\overline{K}}{\rightleftarrows}} ES$$

that the reaction reached an equilibrium. Since there is no reason to consider that equilibrium is necessarily reached, a new treatment was devised by Briggs and Haldane (2). This method does not assume equilibrium in the above reaction but expresses the situation in terms of individual rate constants.

$$E + S \underset{k-1}{\overset{k1}{\rightleftarrows}} ES$$

While we need not consider this theory in any detail here, it should be pointed out that the necessity of saturation of the enzyme by substrate is still necessary in enzyme assay. The reason for this is the fact that the rate equation obtained is of the same form as the Michaelis-Menten equation; where $\overline{K}$ is now replaced by a combination of rate constants.

It should be emphasized that the above discussion has considered only the effect of enzyme and substrate concentrations, and the other variables which can affect the rate have been assumed to be constant. Some of these other variables are:

a) Temperature

b) pH

c) Ionic strength (salt concentration)

Thus the medical technologist must be especially careful to hold these other factors as close to constant as humanly possible in order to be certain that the rates observed in all cases are due only to differences in enzyme concentrations and not to some other effect.

Some possible difficulties—

1) Substrate Inhibition

In this situation the reaction is actually inhibited by very high concentrations of substrate (fig. 2); thus we cannot run our conventional assay procedures at high substrate concentration.

2) Unknown Inhibitor Already Present in Biological Sample

This situation may be true in many biological fluids. Thus, we may mistake low inhibitor concentration for high enzyme concentration.

3) Mixture of Enzymes Having Similar Activity

This problem is generally not of too much importance in clinical work since diagnosis is usually dependent on total activity.

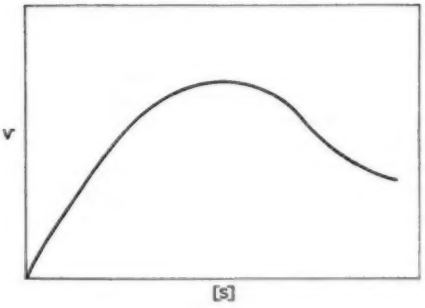


Fig. 2.

It is possible to handle the experiments and data in such a way as to circumvent some of these difficulties; however, such methods are beyond the scope of the present discussion.

It is hoped that this fundamental review of basic principles will illustrate the desirability of understanding these principles and their application to existing enzyme assay methods as well as to the development of new methods as they become necessary.

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A STANDARD AND CONTROL FOR URIC ACID*

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INTRODUCTION

The procedure and reagents in the determination of uric acid can be checked by running several standards.

There are three methods by which the standards can be set up. They are: (a) American College of Pathology, (b) Standard from Archibald's Method, and (c) Pooled Serum as a Control.

METHODS

American College of Pathology. Instructions are enclosed with each package of vials that are received. The solution is made up fresh on the day of use.

Standard from Archibald's Method. A stock solution was made up according to Archibald's modification of Kern-Stransky determination of serum uric acid. From the stock solution two working standards (A and B) were made up. These two standards were made to set up a graph from which the miligram per cent uric acid could be read directly in conjunction with the per cent transmittance.

Standard Working Solution A. 0.1 milligrams per cent of uric acid or 0.000001 grams of uric acid. Place 1.0 cc. of the stock solution into a 1000 cc. volumetric flask and dilute to mark with distilled water.

Standard Working Solution B. 0.05 milligrams per cent of uric acid or 0.0000005 grams of uric acid. Place 0.5 cc. of the stock solution into a 1000 cc. volumetric flask and dilute to mark with distilled water.

From the two working standard solutions the following dilutions were made.

Standard Working Solution A.
A1 5.0 cc. of standard working solution A.
A2 4.0 cc. of standard working solution A and 1.0 cc. of distilled water.
A3 3.0 cc. of standard working solution A and 2.0 cc. of distilled water.
A4 2.0 cc. of standard working solution A and 3.0 cc. of distilled water.
A5 1.0 cc. of standard working solution A and 4.0 cc. of distilled water.

Standard Working Solution B.
B6 5.0 cc. of standard work solution B.
B7 2.5 cc. of standard working solution B and 2.5 cc. of distilled water.
B8 1.0 cc. of standard working solution B and 4.0 cc. of distilled water.
B9 0.5 cc. of standard working solution B and 4.5 cc. of distilled water.

The above dilutions were used as filtrates according to Archibald's modification of the Kern-Stransky determination of serum uric acid.

When plotted on semi-log graph paper the two working standards should give a straight line which, however, does not necessarily pass through the point of zero concentration. A standardization graph should be done each time a new stock solution is made up.

Both an unknown and a standard working solution lying within the

*Read by title, 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959.

normal human range (2% to 4% are run according to Archibald's procedure.

Pooled Serum as a Control. Besides the above working standard a pooled serum in vials can be used as a control. This serum which is pooled each day is a clear and non-hemolyzed serum obtained from the blood bank and the blood chemistry department. When 300 to 350 cc. of frozen serum has been collected, it is then thawed, mixed thoroughly and filtered. After filtering process, it is mixed again and put into vials.

Several of these vials are then taken at random to be tested and the balance refrozen. To insure a complete mixture of the proteins in the serum, care must be taken to be sure that it is mixed throughly. The serums are then treated like unknowns to find the per cent transmittance using the uric acid procedure. If these random samples agree within a certain range, the pooled serum can then be used as a control.

RESULTS

When the above dilutions are used as filtrates, the results represent a 1:20 dilution of the serum. This is comparable to the filtrate from serum with a uric acid concentration of 1:20 times the theoretical calculations.

Dilutions	Theoretical Calculations	As a 1:20 Filtrate
A1	0.5 mgs. %	10.0 mgs. %
A2	0.4 mgs. %	8.0 mgs. %
A3	0.3 mgs. %	6.0 mgs. %
A4	0.2 mgs. %	4.0 mgs. %
A5	0.1 mgs. %	2.0 mgs. %
B6	0.25 mgs. %	5.0 mgs. %
B7	0.125 mgs. %	2.5 mgs. %
B8	0.05 mgs. %	1.0 mgs. %
B9	0.025 mgs. %	0.5 mgs. %

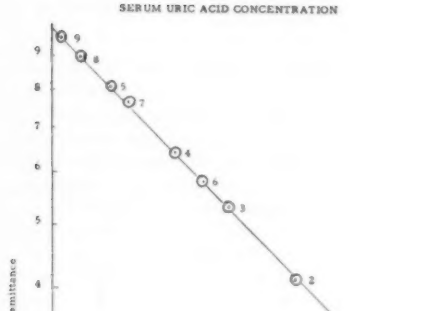
(Graph)

Dilutions	% Transmittance	Mgs. % of Uric Acid
A1	31.5	9.75
A2	42.0	7.25
A3	53.0	5.3
A4	64.0	3.7
A5	81.0	1.8
B6	58.0	4.55
B7	76.5	2.3
B8	90.0	0.9
B9	96.0	0.3

REMARKS

In setting up a uric acid concentration graph, it is necessary to plot at least two points to determine a straight line. This straight line will permit the medical technologist to read the percent uric acid directly from the graph.

The reason for plotting at least two points is the uncontrollable variables which do not necessarily allow the line to pass through the point of zero uric acid concentration.



Summary

By using two working standards to set up the concentration graph and the pooled serum control, flaws in the procedures, reagents, results and technique will be detected.

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DETECTION OF RHEUMATOID FACTOR(S) By ALLIGATOR ERYTHROCYTE HEMAGGLUTINATION*†

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It is almost ironical that the sensitized erythrocyte of a reptile can be effectively used in serological tests for rheumatoid arthritis. The earliest known case of multiple arthritis is in a fossil swimming reptile—the platecarpus—which is calculated to have lived approximately 100 million years ago.[1] Medical historians noted that the work shops of the 17th century alchemists were invariably adorned with stuffed relatives of the alligator and the crocodile. Those preserved reptiles testified to the universal belief of the crocodile's usefulness in the treatment of human ailments and that research into health as well as wealth was ". . . a prime goal of alchemy.[2] Antibody-sensitized erythrocytes of mammalian species,[3, 4, 5, 6, 7] as well as human[5, 6, 8] have been used in hemagglutination tests for rheumatoid factor. Only recently[9, 10] have the red blood cells of a reptile, the alligator *(Alligator mississipensis)* been used in such serological tests. It is the purpose of this report to give the origin and current status of the alligator erythrocyte hemagglutination test.

During basic studies of heteroagglutinins in lower vertebrate sera, it was observed that alligator serum had natural antibodies to human O, A, and B agglutinogens as had been reported earlier by Bond.[11] Alligator erythrocytes did not reveal agglutinogens A, B, C, D, E, c, e, M, N, Kell and Duffy when tested by standard saline, albumin, and Coombs techniques with potent grouping sera. However, it was observed that human sera with rare exceptions lacked heterophile antibodies to alligator erythrocytes, under standard conditions. The question was raised, as to in what diagnostic laboratory procedure might alligator cells be used effectively. The answer—the sensitized red cell test for rheumatoid arthritis. The use of sheep cells in serological tests for rheumatoid arthritis had necessitated prior absorption of heterophile antibodies with packed sheep erythrocytes, or the establishment of a differential agglutination titer.

Erythrocytes were obtained in ammonium-potassium oxalate from blood collected by cardiac puncture from young adult alligators. The same individual alligators have been used as blood donors for more than 2½ years and are in good health. In addition, the cells were found to have extraordinary durability to osmotic and mechanical stress, as well as the capacity to withstand storage at 4°C for more than 30 days.[10] Hemagglutination phosphate buffer (Difco) was used to prepare a 2 per cent thrice washed suspensions of cells for immunization and test antigen. Alligator erythrocytes sensitized with inactivated rabbit anti-alligator erythrocyte serum—one half of the minimum antibody concentration with which macroscopically visible agglutination was obtained —were agglutinated after incubation for 30 minutes at 37°C with the

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inactivated sera of patients with rheumatoid arthritis. Recent study[12] indicates that the sera of 80 per cent of subjects with active rheumatoid arthritis will agglutinate alligator erythrocytes sensitized with homologous rabbit antibodies. Since no heterophile antibodies are present to alligator cells, there is no need for prior absorption, of the patient's serum nor for differential agglutination titers. Sera of patients with osteoarthritis, gout, rheumatoid spondylitis, and and dermatomyositis, and lupus erythematosus give negative results. The alligator erythrocyte hemagglutination system has appeared to be more sensitive and specific than the sensitized sheep cell system.[10] There is a favorable correspondence of alligator cell tests with the results of latex particle fixation (Hyland slide method) and the original Singer-Plotz method.[17]

Detailed instructions for the performance of the alligator erythrocyte hemagglutination test follow:

1. *Wash erythrocytes* three times with phosphate buffer (Difco) and prepare a *2%* suspension.
2. Initially *inactivate amboceptor* (undiluted) for 30 minutes at 56°C; thereafter merely keep under refrigeration.
3. Carry out an *amboceptor titration* with the previously prepared 2 per cent suspension of alligator cells as follows:
   a. Dilute amboceptor in buffer ½, ¼, ⅛ to 1/512, having a total volume of 0.3 ml. of the dilution per tube.
   b. To each of the above tubes containing the amboceptor dilution add 0.6 ml. of the washed cell suspension. Mix.
   c. Incubate at 37°C for 30 minutes.
   d. Centrifuge 1 to 2 minutes at 500 RPM and read *macroscopically*.
   e. Select the dilution beyond the last tube containing definite agglutination as your cells sensitizing dilution of amboceptor.
   Example: If ¼ = 2 plus, ⅛ = 1 plus, 1/16 = negative, then 1/16 = cell sensitizing dilution.
4. **Sensitization of erythrocytes**
   a. Prepare enough sensitizing dilution of amboceptor in buffer to do all tests required. Add 2 volumes of the 2 per cent suspension of washed alligator cells to 1 volume of the cell sensitizing dilution. Mix. Incubate at 37°C for 15 to 30 min. Wash cells 3 times with phosphate buffer and resuspend to 2 per cent concentration.
5. **Test Procedure:**
   a. Transfer 0.1 ml. of the above sensitized cell mixture to each 12 x 75 mm. test tube.
   b. Centrifuge and decant supernate.
   c. Add 0.2 ml. of the patient's serum to the sediment. Mix.
   d. Incubate at 37°C for 30 minutes.
   e. Centrifuge at 500 RPM for 1 to 2 minutes. Read *macroscopically*.

Controls:

Sediment of 0.1 ml. of *2%* suspension of unsensitized cells
plus
0.2 of patient's serum

Sediment of 0.1 ml. of *2%* suspension of sensitized cells
plus
0.2 ml. of phophate buffer

6. **Method of reporting results**

Macroscopically visible agglutination = *Positive*
(Can be graded 1, 2, 3, or 4)
No agglutination = *Negative*

Note: The results can be quantitated by doing the test with serial dilutions of the patient's serum.

Sensitized alligator cells have been stored in phophate buffer for as long as 2 weeks at 4°C.

Modifications of the method are under study.[13] It has been observed that the sensitized alligator cells are suitable for use with cold precipitates,[14] or Ziff[15] euglobulin fractions of serum. Complex inhibition procedures have proved relatively simple to perform with sensitized alligator cells. The cells are being employed in current research as tools to study the nature of agglutination activating factors in rheumatoid arthritis and also in certain non-rheumatic diseases. They have also proved invaluable in fundamental studies of effects of proteolytic enzymes on hemagglutination.[16]

Red cells of the caiman *(Caiman spp.)* of Central and South America and of the crocodile *(Crocodylus spp.)* are serologically similar and can be readily substituted for alligator cells. Alligator farms and cooperative zoos are possible sources of erythrocytes. However, enough blood to do routine tests can be readily obtained from "pet" size alligators by cardiac puncture with a 5cc. syringe and 20 gauge hypodermic needle. Amboceptor is not yet commercially available, but limited amounts can be obtained free on request from the author.

Summary

The origin, development and technique of a practical sensitized alligator erythrocyte hemagglutination test for determination of rheumatoid factor(s) has been described. There is a virtual absence of heterophile antibodies in human serum to alligator cells. In addition, the cells possess an extraodinary durability. Within the collagen disease category, the sensitized alligator cell test possesses a favorable degree of specificity and sensitivity for sera of patients with rheumatoid arthritis.

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THE URINE CULTURE

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Introduction

In civilian medical practice, where the patient has to pay for laboratory analyses, bacteriological procedures are not as often employed as might be desirable. However, students of statistics will have no difficulty in finding that one of the more frequently requested bacteriological procedures is the urine culture.

In the age of antibiotics there is, for obvious reasons, a definite need not only to determine the infecting organism but also to find the antibiotic which will demonstrate the highest therapeutic efficiency. There are very good reasons why so many physicians treat the patient by "trial and error." For one, it is the prohibitive cost of bacteriological examinations charged by many laboratories, but most of all, it is the time many bacteriologists require before they are able to render a report of their findings.

While the textbooks on medical bacteriology have adequate descriptions of the organisms responsible for disease and infection, they give but little guidance to the student of bacteriology about securing the specimen. It is the purpose of this paper to describe techniques which will allow the medical bacteriologist to report the results of urine cultures within 24 hours and provide at the same time the antibiotic and chemotherapeutic agent most suitable for therapy.

Procedure

In Bacteriology, the results obtained in any investigation are as good, certainly never better as, the care which has been exercised in obtaining the specimen to be cultured. It is therefore important that the bacteriologist be instrumental in formulating techniques and procedures to this end.

A specimen submitted in a urine bottle filled to the brim, over which a paper towel or a 4 x 4 sponge has been draped, or a test tube with 3 ml of urine, topped with a freshly plucked piece of absorbent cotton, will greatly eliminate the possibility of culturing the true flora present in the urinary tract of the patient. A standard urine bottle which has been closed with a gauze covered cotton plug, similar to the type which is employed in the sterilization of Ehrlenmeyer flasks, is most suitable for the purpose. While in storage this bottle is further protected by a wrapper of aluminum foil. The bottle, of course, is sterilized by autoclaving.

The specimen must be collected under the most exacting sterile conditions when the patient has a full bladder. The specimen from females must be obtained by catheterization. Male patients must be scrubbed carefully and the specimen collected under strict sterile precautions. Catheterization of males is not necessary, but it is important that the foreskin of uncircumcised patients remains retracted during the collection. The first 2-4 ml of the urine voided are discarded and the following 20-30 ml are collected. Regardless of whether the infection is an anterior or posterior one, the first specimen will always have the offending microorganism.

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The specimen should be received by the bacteriologist as expeditiously as possible. Observing established procedures which include flaming during transfer, approximately 15 ml of the well mixed specimen are decanted into a sterile culture tube and the specimen centrifuged for 20 minutes at 2000-3000 RPM. After centrifugation the supernatant urine is poured off as completely as possible, and one-third of a blood agar plate solidly inoculated with a sterile swab which has been heavily infected with the sediment. A tube of Thioglycollate broth is inoculated with a second swab. The blood agar plate is then streaked with a platinum loop in such a manner that well isolated colonies can be obtained in the thinned out areas of the blood plate. Two wide spectrum antibiotic discs are spaced well separated in the area of the plate which has been heavily inoculated with the infected swab. The blood agar plate as well as the Thioglycollate broth are placed in the incubator set at 35° C.

By far the greatest number of organisms isolated from the urinary tract belong to the Coli-Aerogenous group. These organisms grow rapidly and it is not unusual that the first signs of growth can be perceived after 5-6 hours of incubation. An examination of the blood plate at this time will allow the following observations:

1. Is the growing organism susceptible to the antibiotics selected? This is important to the physician.
2. Is the organism Gram positive or Gram negative? This is important to the physician and to the bacteriologist.

A preliminary report can be made at this time which may guide the physician in his course of therapy. If the organism is Gram negative and shows a tendency of swarming, placing filter paper in the bottom of the Petri dish to which 10-12 drops of 99 percent Methyl Alcohol have been added, will in many instances, prevent further swarming. If the culture appears to be pure (the majority of sterilely collected urine cultures are), a Kligler's Iron-Agar slant, Urease media and the biochemicals of the IMVIC (Indole, Methyl Red, Voges-Proskauer, Citrate) should be inoculated before closing shop for the day. If the organism does not show susceptibility to the two antibiotics planted with the specimen. another blood plate for the whole range of antibiotics and Mueller-Hinton media for the Sulfonamides can then be seeded. By following these recommendations it will be possible in many instances to report to the physician within 24 hours:

1. The Genus and Species of the organism.
2. Qualitatively the degree of the infection based on the amount of the flora appearing on the blood plate.
3. The antibiotics or chemotherapeutic agents to which the organism is susceptible.

If the Kligler's Iron-Agar reactions indicate that the organism isolated does not belong to the Coli-Aerogenous group, the Urease reaction will show if it is a Proteus. The biochemicals of the IMVIC test (Indole, Methyl Red, Voges-Proskauer, Citrate) together with the reactions of the Kligler's Iron-Agar will allow the identification of such organisms as Pseudomonas aeruginosa, Klebsiella pneumoniiae, Alkaligenes fecalis the Paracolons, Paracolobactra, and even the Salmonella. Most Gram positive organisms invading the urinary tract, such as Staphylococcus

and Streptococcus species can be identified from the blood plate without additional culture work.

It will be worthwhile to reflect that not every organism causing a negative/negative reaction on Kligler's Iron-Agar is necessarily an Alkaligenes fecalis, which is generally not considered a pathogen, but could very well be a Mima species which has been implicated in severe infections. The same thoughtful criteria should be applied in evaluating a diphtheroid-like organism, which may be a Listeria if motile and hemolytic. Yeasts isolated from a urine are not necessarily Saccharomyces, neither are they always Candida albicans.

A smear from the Thioglycollate broth should confirm the flora of the blood plate. However, Thioglycollate broth may show organisms which do not grow initially on solid media and may be bacteria requiring micro-aerophilic or anaerobic conditions for their propagation. Thioglycollate broth apparently has a neutralizing effect on those organisms which due to previous therapy, fail to grow on solid media. It has been found that the invading organism could be recovered from this broth while the blood plates remained sterile.

To the physician investigating his patient for urinary infection the following information is essential:

1. Is there an infection?
2. How severe?
3. To which antibiotic or chemotherapeutic agent does the offending organism respond?
4. Genus and Species of the organism isolated.

However, most important to him is the time element in which this information is available.

Summary

This paper has endeavored to recommend procedures by which the results of urine cultures can be reported, in many cases within 24 hours. Techniques of collection are shown and methods recommended by which the growing organism is used, as soon as it can be detected, to perform the classical procedures of identification of the organism and to investigate susceptibility to antibiotics.

It is projected that the medical bacteriologist has an obligation to his profession to accept and perform examinations only on those specimens which give all indications that they have been collected under the most exacting circumstances. The medical bacteriologist must be instrumental in instructing personnel and suggesting techniques if he feels that those charged with the collection of specimen are not familiar with sterile procedures, as they apply to bacteriology.

It is believed that the physician, who uses urine cultures and other bacteriological cultures only as a "last resort," can be persuaded to use more scientific methods of analysis if reports are rendered within a reasonable period of time.

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